*The Journal of*
Clinical Endocrinology and Metabolism

VOLUME 21 JANUARY, 1961 NUMBER 1
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# PURIFICATION OF PITUITARY GONADOTROPIN FROM URINE OF NORMAL MEN*

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ABSTRACT

Human pituitary gonadotropin (HPG) from urine of normal men was purified in three successive steps. HPG was extracted from urine with kaolin, eluted with ammonium hydroxide, and precipitated with acetone. The acetone precipitate was extracted with 10 per cent ammonium acetate in 70 per cent ethanol. Impurities of this extract were removed by adsorption on diethylaminoethyl cellulose. The partial purification of HPG achieved by these three steps was of the order of 50-fold. The partially purified material had the same biologic characteristics as the initial extract, as determined by the method of biologic fingerprint and by three assay systems: the ovarian-weight method in intact rats, the augmentation reaction with chorionic gonadotropin, and the ventral-prostate method in hypophysectomized rats.

INTEREST in the gonadotropic material of human urine (human pituitary gonadotropin, or HPG) has increased during the past five years because of progress in the use of clinical tests of HPG and because of curiosity regarding its fundamental biologic nature. In order that sufficient amounts of HPG may be obtained for study, large quantities of urine must be processed. Consequently, increasing attention is being given to methods of extracting and purifying the gonadotropic material in the urine of nonpregnant women and of men.

The initial step in processing urine is removal of HPG from the urine by

Received May 21, 1960.

* Aided by grant A-1738, National Institutes of Health, United States Public Health Service.

adsorption. Two procedures have been used chiefly: (a) urine is passed through a zeolite column and the retained HPG is eluted, and (b) kaolin is added to urine and the HPG adsorbed to the kaolin is eluted. The first method yields a product with high specific activity; however, judging from comments of several authors, this method is capricious. The second procedure yields a product of lower specific activity, but it appears to be more reliable; several investigators have used it routinely to prepare HPG in bulk.

The kaolin procedure was used in this laboratory to devise a method for extracting HPG from 90-liter batches of urine (1). The main advantage of this method is that it permits processing, at a leisurely pace, of large quantities of urine which will yield sufficient gonadotropin for biologic studies. However, if HPG is to be prepared in the amounts required for chemical studies, leisurely preparation becomes of secondary importance. The main requirement for chemical studies is rapid extraction or concentration of as much HPG from the urine as possible, so that large quantities can be handled on a daily basis. This primary requirement makes it unnecessary that the specific activity of the crude HPG be as high as possible, since subsequent procedures can be expected to purify the crude concentrate.

For the purpose of preparing large amounts of HPG rapidly, the routine procedure for extracting HPG from clinical specimens of urine was explored for the possibility of adapting it to large quantities of urine. The routine method (2) consists of the following steps: (a) addition of 20 Gm. of kaolin (dry) to a 24-hour or 48-hour specimen of urine at pH 4.5, (b) rapid filtration by an automatic filtration apparatus, (c) washing of the kaolin cake with 2 liters of tap water at pH 4.5, (d) elution of HPG by percolation with 100 ml. of $2M$ $NH_4OH$ followed by 30 ml. of distilled water, and (e) precipitation of HPG from the acidified (pH 5.5) eluate with 2 volumes of acetone. The 66 per cent acetone precipitate is crude HPG and will be referred to hereafter as "control fraction A," or C.F.A. After removal of excess acetone by means of a stream of air, C.F.A. may be dissolved or suspended directly in water and assayed (C.F.A.-1), or it may be dried in the usual manner by washing with ethanol, ethanol-ether and ether, after which it is weighed, stored, and then dissolved in water for assay (C.F.A.-2).

For clinical specimens of urine (1 to 2 liters), this procedure takes two hours. With suitable modifications it has been possible to process large quantities of urine for HPG with satisfactory rapidity. The rapid preparation and further purification of gonadotropic material are the subject of this report.

## MATERIALS AND METHODS

The HPG was extracted from pooled male urine. The source and characteristics of this urine have been described previously (3). The reason for using male urine—which

is of low gonadotropic potency—is its availability. Collection of large amounts is not difficult, and with appropriate measures there is little possibility of contamination with human chorionic gonadotropin (HCG).

The biologic assay of the material has been described fully (3). Special assays will be described in appropriate parts of this paper. Biologic activity is expressed as rat units. The specific activity of any fraction of HPG is expressed as rats units per milligram of HPG. One rat unit (R.U.) is the equivalent of 2.4 mg. of the standard HMG-20A in this general assay method.

The amount of material in any fraction was determined by either of two methods. When large amounts of material were present, as in crude HPG, the material was dried (by the method already described herein) and weighed. When the amount of material was small, protein determinations were made by a suitable modification of the method of Lowry and associates (4). In the latter instance, standards were prepared from material similar to that being studied, and consequently there was excellent agreement between the amount of material determined by protein measurements and the amount of material determined by weighing the dried precipitate.

The efficacy of the purification procedures was determined by expressing the specific activity of any fraction as a multiple of the specific activity of the crude HPG. It is not correct to express the purity of any fraction in terms of its specific activity alone or to make comparisons on this basis of the purity of various products described by different investigators. The reason for this is that the specific activity of any preparation depends on the source of the urine employed. Therefore, if two methods of purification are to be compared and if urines of differing original potency are used, the degree to which the starting material has been purified, not the specific activity of the fraction itself, is the correct basis for comparison.

## RESULTS

### *Preparation of crude HPG (fraction A)*

*Characteristics of the pooled male urine.* In all of the experiments on large batches of male urine (usually 60 liters) from the pool, two control extractions were carried out by the routine procedure. For example, if a 60-liter volume of urine was to be processed, a total of 66 liters of urine was thoroughly mixed. Two 3-liter aliquots were removed and processed by the routine clinical method as controls. The 66 per cent acetone precipitates from these aliquots were dried, weighed, and assayed (C.F.A.-2). During the year 1958, 112 such control extractions were performed. The results are shown in Table 1. The mean yield of solid material was 66.9 mg. per liter of urine, the mean activity was 8.3 rat units (R.U.) per liter, and the mean specific activity was 0.14. These characteristics form the basic reference for all the work to be described in this paper.

*Amount of kaolin.* The changes introduced for effecting rapid preparation of fraction A will be described only in a summary form. Repeated trials showed that the rate of filtration of the kaolin-urine suspension, the rate-limiting step of the entire procedure, was slowed when more than 100 Gm. of kaolin was handled by one chamber of the automatic filtration apparatus. Since four such chambers constituted our equipment, it was evident that no more than 400 Gm. of kaolin could be used for any lot of urine. Since

effective adsorption of HPG depends on a certain critical quantity of kaolin, the quantity of urine that could be processed as a unit was thus dependent on the amount of kaolin used, and this in turn was governed by the rapidity of processing desired. Consequently, a re-investigation was made of the recovery of HPG as a function of the amount of kaolin. In these experiments a total of 12 liters of urine was divided into four 3-liter portions. Varying amounts of kaolin were added in dry form (0.3 to 10 Gm. per liter). This experiment was repeated 10 times (40 assays). It was found that 5 Gm. of kaolin per liter of urine was the minimal amount of kaolin associated with the maximal recovery of gonadotropin. When 5 Gm. of kaolin per liter was used, it was found that satisfactory flow rates could be achieved

TABLE 1. CHARACTERISTICS OF MALE URINE (112 ASSAYS, 336 LITERS)

| Month (1958) | Total liters | No. of assays | Solids (mg./L.) | | Activity (R.U.*/L.) (mean) | Specific activity (R.U.*/mg.) |
|---|---|---|---|---|---|---|
| | | | Mean | Range | | |
| January | 54 | 18 | 57.9 | 42.8– 74.3 | 14.2 | 0.25 |
| April | 39 | 13 | | | 6.9 | |
| May | 9 | 3 | 58.8 | 41.9– 75.6 | 7.2 | 0.12 |
| June | 39 | 13 | 83.4 | 33.3–113.4 | 13.1 | 0.16 |
| July | 48 | 16 | 75.4 | 47.5– 99.2 | 8.4 | 0.11 |
| August | 15 | 5 | 88.1 | 82.7– 93.4 | 5.0 | 0.06 |
| September | 36 | 12 | 46.7 | 41.6– 51.8 | 7.3 | 0.16 |
| October | 60 | 20 | | | 6.8 | |
| November | 30 | 10 | 64.9 | 43.3– 81.4 | 7.7 | 0.12 |
| December | 6 | 2 | 59.6 | 56.9– 62.2 | 6.5 | 0.11 |
| Mean | | | 66.9 | | 8.3 | 0.14 |

* R.U. = rat units.

when a total of 15 liters of urine was processed. Thus 75 Gm. of kaolin per filtration chamber was employed. With four chambers available, 60 liters of urine could be processed as a convenient unit.

*Physical state of kaolin.* When dry kaolin is added to urine, a few small clumps are formed. If the kaolin is present in excess, this clumping is of no consequence, but when the amount of kaolin is minimal, clumping may lead to incomplete recovery of HPG. Various trials were made with kaolin emulsified (by a Waring blendor) in urine, water, acetone, ethanol, and various mixtures of these substances, but none was satisfactory. The method recommended by earlier workers of suspending kaolin in water before addition to the urine was finally used. The kaolin (75 Gm.) was mixed with a stirring rod into 200 ml. of acidified tap water (pH 4.5). The suspended kaolin was poured into the vessel containing 15 liters of urine. More water was added to the remaining kaolin clumps, and the clumps were restirred. This procedure was continued until all the kaolin was suspended; as a result, clumps were not formed in the urine.

It was found convenient to begin the procedure of obtaining crude HPG late in the afternoon. The urine (60 liters) was adjusted to pH 4.5 with glacial acetic acid and placed in four plastic vessels, 15 liters per vessel. The kaolin (75 Gm.) was suspended and added to each vessel; it was allowed to settle by gravity. Next morning the supernatant urine was removed by siphon. By gently tilting the vessel, almost all the urine could be removed. This procedure obviated the need for filtration of the urine, and with suitable scheduling allowed orderly daily preparation. After siphoning, celite (a 50-ml. beakerful, approximately 11 Gm.) was added to each vessel, and the contents were stirred. The contents of the vessel were transferred quantitatively (with the aid of small washes of water at pH 4.5) to the filtration chamber and rapidly filtered. This yielded a cake of kaolin-celite in each of the four filtration chambers.

*Washing the kaolin cake.* Prolonged washing of the kaolin leads to a product with higher specific activity. However, for rapid preparation of HPG, prolonged washing of the cakes was not attempted. Instead, each cake was washed by passing 2 liters of tap water (pH 4.5) through the chamber. This step, which ..as accomplished in about twenty minutes, removed adherent urine.

*Elution.* Studies of the elution of HPG from the kaolin-celite cakes will not be described in detail. It was found that recovery of HPG was inversely proportional to the amount of kaolin used, but that the specific activity was higher when large amounts of kaolin were used. This presumably resulted from more effective removal of HPG than of inert material when large amounts of kaolin were present. With a cake of constant size (75 Gm.), the greatest amount of HPG could be removed by grinding each cake in a blendor with 200 ml. of 2*M* $NH_4OH$. The cake was removed from the chamber and placed in a Waring blendor with the $NH_4OH$. The speed of the blendor was reduced or the blades were inverted to reduce shearing action, and mixing was allowed to continue for one minute. The resulting emulsion was placed in the filtration chamber, which had been prepared with a bed of 6 Gm. of celite, and the eluate was collected. The cake was again removed and the grinding process repeated with a fresh 200 ml. of $NH_4OH$; then the filtration process was repeated. This filtrate was added to the first filtrate. The combined eluate (400 ml.) was clear and mahogany-colored.

*Precipitation of HPG.* Glacial acetic acid (about 40 ml.) was added, with stirring, and the solution was brought to pH 5.5. Distilled water was added to bring the volume to 450 ml. (by marking on a suitable vessel), and 2 volumes (900 ml.) of acetone were added. All four cakes were treated in a similar fashion, yielding 5400 ml. of the 66 per cent acetone suspension. Precipitation occurred rapidly; it was facilitated by storage overnight at

5° C. The 66 per cent acetone precipitate was then handled in the following manner:

1. The crude HPG was centrifuged and dried (fraction A-2, for pilot studies).

2. The 66 per cent acetone suspension was mixed with celite (one half of a 50-ml. beakerful) and filtered quickly through one chamber (wet fraction A-3, for proceeding immediately to further purification).

3. The cake of celite and crude HPG was dried by passing air or 200 ml. of acetone through the cake. The cake was removed, allowed to dry further, and powdered (dry fraction A-4, for stockpiling before subsequent purification).

*Preparation of fraction B*

*Preliminary experiments.* In these trials a 12-liter sample of pooled male urine was distributed into four 3-liter aliquots and processed by the routine clinical method in order to obtain 66 per cent acetone precipitates (C.F.A.-1). Two of these wet precipitates were dried, weighed, and assayed as controls (C.F.A.-2), and two were used for experimental trials. The experiments consisted of extracting the wet precipitates with solutions of ammonium acetate ($NH_4Ac$) of various concentrations and of ethanol (EtOH) of various concentrations; with various mixtures of $NH_4OH$ and $NH_4Ac$; with various mixtures of $NH_4Ac$ and EtOH; and with various mixtures of $NH_4OH$, $NH_4Ac$, and EtOH. Eighty-five experiments entailing 387 assays were performed.

It was found that 10 per cent $NH_4Ac$ in 70 per cent EtOH was the best extractant. The two experimental aliquots were extracted twice with 7 ml. of 10 per cent $NH_4Ac$ in 70 per cent EtOH. The combined extract was mixed with 2 volumes of about 10 per cent $NH_4Ac$ in absolute EtOH. The precipitate which formed was dried, weighed, and assayed. In Table 2 are listed the results of two trials, one consisting of three runs on one batch of urine and another comprising five runs on another batch of urine. The mean content of solids of the extracted material was 6.0 mg. per liter, the mean activity was 6.9 R.U. per liter, and the mean specific activity was 1.2. This represents one-tenth of the solid material and 80 per cent of the activity present in fraction A. The purification achieved was ninefold.

*Large-scale preparation.* Approximately 60 runs with 60 liters of urine per run and approximately 290 assays were made to ensure the consistency of fraction B prepared in bulk.

The urine was processed as fraction A-3; that is, the 66 per cent acetone precipitate-celite suspension was passed through a filtration chamber. Acetone (200 ml.) was then flushed through the celite cake, and the cake was dried by passing air through the chamber. The cake was removed, al-

TABLE 2. EXTRACTION WITH 10 PER CENT $NH_4Ac$ IN 70 PER CENT EtOH AND PRECIPITATION AT 90 PER CENT EtOH

| Number | Control* | | | | | | Experimental* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mg./L. | R.U./L. | S.A. | Mg./L. | R.U./L. | S.A. | Mg./L. | R.U./L. | S.A. | Mg./L. | R.U./L. | S.A. |
| 1 | 46.6 | 11.3 | 0.24 | 46.4 | 7.1 | 0.15 | 5.3 | 5.9 | 1.11 | 6.2 | 9.3 | 1.50 |
| 2 | 43.8 | 7.0 | 0.16 | 41.6 | 9.3 | 0.22 | 3.3 | 5.9 | 1.79 | 3.7 | 4.7 | 1.27 |
| 3 | 51.8 | 4.7 | 0.09 | 50.2 | 10.0 | 0.20 | 4.9 | 6.7 | 1.37 | 5.4 | 8.3 | 1.54 |
| 4 | 97.3 | 6.6 | 0.07 | 96.2 | 6.2 | 0.06 | 5.2 | 5.3 | 1.02 | 4.4 | 5.4 | 1.23 |
| 5 | 43.0 | 8.6 | 0.20 | 51.6 | 7.2 | 0.14 | 6.8 | 5.1 | 0.75 | 8.8 | 6.5 | 0.74 |
| 6 | 51.5 | 7.7 | 0.15 | 57.4 | 6.7 | 0.12 | 8.5 | 7.3 | 0.86 | 9.0 | 8.9 | 0.99 |
| 7 | 93.5 | 15.0 | 0.16 | 97.4 | 11.3 | 0.12 | 6.7 | 5.2 | 0.78 | 6.0 | 9.2 | 1.56 |
| 8 | 88.3 | 6.7 | 0.08 | 86.4 | 10.4 | 0.12 | 6.1 | 7.3 | 1.20 | 6.1 | 9.4 | 1.54 |
| Mean | 65.2 mg./L.<br>8.5 R.U./L.<br>0.14 S.A. | | | | | | 6.0 mg./L.<br>6.9 R.U./L.<br>1.2 S.A. | | | | | |

* R.U. =rat units; S.A. =specific activity.

lowed to dry further in air, and ground. The powder was placed in the Waring blendor with 120 ml. of 10 per cent $NH_4Ac$ in 70 per cent EtOH. Grinding was accomplished as described for grinding of the kaolin cakes. The suspension was placed in the filtration chamber and filtered. The filtrate was then returned to the chamber, passed through the cake, and collected. Fresh extractant (120 ml.) was added to the chamber, flushed through the cake, and collected. The combined filtrate was mixed with 2 volumes of 10 per cent $NH_4Ac$ in 100 per cent EtOH and placed in the refrigerator overnight. The precipitate was centrifuged, dried, weighed, and stored (fraction B). All urine was processed to this point and stockpiled. In Table 3 are shown the results of the last 11 of 40 runs, which were handled as described or with minor modifications. The mean yield of solids from 60 liters of male urine was 295 mg. The mean concentration was 4.8 mg. per liter. The mean specific activity was 1.53. The mean activity per liter was 7.3 (1.53×4.8). When these values are compared with those in Table 1, it is seen that 7 per cent of the solids originally present were ex-

TABLE 3. CHARACTERISTICS OF FRACTION B
(60 LITERS MALE URINE)

| Run | Total weight of solids (mg.) | Solids (mg./L.) | Specific activity |
|---|---|---|---|
| 1 | 348 | 5.8 | 1.02 |
| 2 | 283 | 4.7 | 2.00 |
| 3 | 335 | 5.6 | 1.34 |
| 4 | 341 | 5.7 | 1.17 |
| 5 | 282 | 4.7 | 1.69 |
| 6 | 310 | 5.2 | 2.11 |
| 7 | 274 | 3.5 | 2.48 |
| 8 | 337 | 5.6 | 1.02 |
| 9 | 254 | 4.2 | 1.62 |
| 10 | 263 | 4.4 | 1.08 |
| 11 | 219 | 3.7 | 1.31 |
| Mean | 295 | 4.8 | 1.53 |

tracted (4.8 mg./L. ÷66.9 mg./L.), and 88 per cent of the activity was retained (7.3 R.U./L. ÷8.3 R.U./L.). The purification achieved over the crude material was 10-fold (1.53 R.U./mg. ÷0.14 R.U./mg.).

*Preparation of fraction C*

About 265 experiments were performed with fraction B to determine the most appropriate conditions for purification of HPG by diethylaminoethyl cellulose (DEAE-C). In the course of these experiments, fraction B was assayed 82 times. The assay consisted of injecting 10 mg. of fraction B into each of 2 rats used as controls in the various experiments. The mean potency of 10 mg. of fraction B was 11.9 R.U., or a specific activity of 1.2 R.U./mg.

Preliminary experiments (which will not be detailed) indicated that the HPG of fraction B could be adsorbed from water at neutral or acid pH by DEAE-C. Fraction B (20 mg.) was dissolved in 7 ml. of water at any desired pH and added to varying amounts of dry DEAE-C in proportions of HPG:DEAE-C of 1:1, 1:3, and 1:10. The suspension was stirred and centrifuged. The DEAE-C was washed with another 7 ml. of water at the pH under study and recentrifuged. The combined supernatant solution was filtered quickly through coarse filter paper to remove strands of cellulose, and then it was brought to 16 ml. with water. A small portion (0.5 ml.) was removed for protein analysis, and the remainder was assayed for gonadotropin. The amount of solids adsorbed to DEAE-C varied in direct proportion to the amount of cellulose used. At a ratio of HPG:DEAE-C of 1:10, adsorption was complete. The solutions after treatment with DEAE-C were water-clear, demonstrated no HPG activity, and contained negligible amounts of protein. The HPG was retained on the DEAE-C.

The DEAE-C was treated with a variety of substances to determine whether the retained HPG from 20 mg. of fraction B could be recovered. In these experiments the DEAE-C to which HPG was adsorbed was washed twice with 5 ml. of the various solutions. In some cases (in which the eluants were mixtures of $NH_4Ac$ and EtOH at various pH's) the combined supernatant fluid was neutralized and brought to a concentration of 90 per cent EtOH. The resulting precipitate was dissolved in water and assayed. In other cases (inorganic bases) the combined supernate was neutralized, brought to a total volume of 16 ml., and assayed. In still other instances (dilute inorganic salts) the combined supernate was diluted to a total volume of 16 ml. and assayed. In all cases, eluting solutions were brought to 16 ml., and a small aliquot was removed for protein analysis. Results of experiments in which 13 eluants were tried are shown in Table 4. As a gauge of the efficacy of removal of HPG, it should be recalled that 24 R.U. (20 mg.×1.2) was adsorbed on DEAE-C and that 20 mg. of protein was

TABLE 4. ELUTION OF HPG IN FRACTION B (20 MG.) FROM DEAE-C (200 MG.)

| Eluant | Results* | | |
|---|---|---|---|
| | T.P. | T.A. | S.A. |
| 10 per cent $NH_4Ac$ | 10 | 18 | 1.8 |
| 10 per cent $NH_4Ac$ in 70 per cent EtOH (pH 7.0) | 6<br>10 | 9<br>13 | 1.5<br>1.3 |
| 10 per cent $NH_4Ac$ in 66 per cent EtOH (pH 9.5) | 8 | 12 | 1.5 |
| Borate buffer—1 per cent NaCl (pH 9.5) | 12 | 10 | 0.8 |
| 0.1*M* $NaH_2PO_4$ buffer—1 per cent NaCl (pH 7.0) | 12 | 14 | 1.2 |
| 1.0*M* $NH_4OH$ | 8<br>6 | 12<br>14 | 1.2<br>1.5 |
| 0.01*N* NaOH<br>0.10*N* NaOH | 9<br>14 | 11<br>8 | 1.2<br>0.6 |
| 1 per cent NaCl | 14<br>13 | 24<br>21 | 1.7<br>1.6 |
| 1 per cent $Na_2CO_3$ | 13 | 25 | 1.9 |
| 1 per cent $NaHCO_3$ | 3<br>2 | 13<br>10 | 4.3<br>5.0 |
| 1 per cent NaAc | 4<br>3<br>3 | 13<br>8<br>9 | 3.3<br>2.7<br>3.0 |
| 1 per cent $NH_4Ac$ | 3<br>3 | 10<br>10 | 3.3<br>3.3 |

* T.P. = total protein (mg.); T.A. = total activity, rat units (R.U.); S.A. = specific activity (R.U./mg.).

used. As seen in Table 4, variable amounts of protein and hormone were removed from the DEAE-C. The resulting specific activity was not appreciably higher than that of the starting material. NaCl removed almost all of the HPG but also removed considerable protein, hence providing little purification. $NaHCO_3$ and NaAc removed the least amount of protein and left about half of the activity. This resulted in fourfold purification. However, it was not considered desirable to sacrifice half of the total activity to achieve a fourfold purification at this stage and, therefore, experiments in the reverse direction were undertaken.

The reverse direction involved treating solutions of fraction B with DEAE-C so that the HPG would not be adsorbed under conditions in which considerable impurity might be removed. The data in Table 4 and information from the literature (see under Comment) indicate the importance of the sodium ion in regulating the retention or nonretention of HPG by DEAE-C. Accordingly, fraction B (20 mg.) was dissolved in 7 ml.

TABLE 5. SUPERNATE AFTER BATCHWISE TREATMENT OF FRACTION B (20 MG.) WITH VARIOUS AMOUNTS OF DEAE-C

| Per cent NaCl in 0.1*M* $NaH_2PO_4$ (pH 7.0) | Proportions of fraction B:DEAE-C (mg.)* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1:1 | | | 1:3 | | | 1:10 | | |
| | T.P. | T.A. | S.A. | T.P. | T.A. | S.A. | T.P. | T.A. | S.A. |
| 1.0 | 20 | 26 | 1.3 | 19 | 24 | 1.3 | 15 | 19 | 1.3 |
| 0.3 | 17 | 16 | 0.9 | 15 | 30 | 2.0 | 13 | 21 | 1.6 |
| 0.1 | 17<br>19 | 15<br>22 | 0.9<br>1.2 | 13<br>16 | 14<br>24 | 1.1<br>1.5 | 11<br>11 | 11<br>24 | 1.0<br>2.2 |
| 0.03 | — | — | — | — | — | — | 10 | 28 | 2.8 |
| 0.01 | — | — | — | — | — | — | 9 | 18 | 2.0 |
| Water | 12 | 15 | 1.3 | 5 | 13 | 2.6 | 0 | 0 | 0 |

* T.P. = total protein (mg.); T.A. = total activity, rat units (R.U.); S.A. = specific activity (R.U./mg.).

of solutions of varying concentration of NaCl in 0.1*M* $NaH_2PO_4$ at pH 7.0. Varying amounts of DEAE-C were added. After mixing and centrifuging, the DEAE-C was again stirred with 7-ml. portions of the solutions. The combined supernate was brought to a volume of 16 ml., analyzed for protein, and assayed. The data in Table 5 show that the greater the proportion of DEAE-C to fraction B, the greater was the amount of inert protein removed from solution. A ratio of 10:1 (DEAE-C:B) was most favorable. At any ratio the amount of protein adsorbed was increased as the per cent of NaCl was decreased. HPG was not adsorbed to any extent in any of the solutions containing between 0.01 and 1 per cent NaCl in 0.1*M* phosphate buffer.

In Table 6 are listed further data used to determine conditions wherein the maximal amount of protein could be removed with the least adsorption of HPG. In effect, the concentrations of sodium represented in Table 6 bridge the gap between 0.01 per cent NaCl (Table 5) and the control (water). Various dilutions of 0.1*M* $NaH_2PO_4$ were made and the type of experiment already described was performed. Studies were made at two pH values—7.0 and 4.6. In Table 6 it can be seen that 0.05*M* buffer at pH 7.0 allowed maximal adsorption of nongonadotropic protein with minimal adsorption of HPG. The specific activity of the nonretained HPG was 4.0, representing a considerable purification that can be accomplished with little effort.

For the preparation of larger amounts of fraction C, 1 Gm. of fraction B was dissolved in 100 ml. of 0.05*M* phosphate buffer at pH 7. A clear, black solution resulted. A 10-Gm. portion of DEAE-C was stirred into 200 ml. of 0.05*M* phosphate buffer at pH 7 and filtered through coarse paper on a

Buchner funnel. A tampon was used to squeeze out excess buffer from the pad of DEAE-C. The cellulose pad was then added to the beaker containing the 100-ml. solution of fraction B. The cellulose was stirred until it became uniformly brown. The brown DEAE-C mush was then filtered through coarse filter paper on a Buchner funnel, and excess fluid was squeezed out with a tampon. A total of 100 ml. of 0.05*M* phosphate buffer at pH 7 was then poured on top of the pad and allowed to percolate through. Intermittent suction was applied as an aid to filtration. After this buffer wash passed through the pad, another 100-ml. wash was performed in the same manner. Excess fluid was again squeezed out by means of a tampon and collected in the filtrate. The filtrate was a clear, light-yellow solution. Glacial acetic acid was added dropwise until a pH of 4 was obtained. Sufficient NaCl was added to make a 1 per cent solution. Four volumes of 95 per cent EtOH were added and the resulting mixture was allowed to stand at room temperature overnight. Next morning, the white precipitate which had formed was filtered on a Buchner funnel through Whatman No. 50 paper. When the last of the fluid passed through the paper, the precipitate

TABLE 6. SUPERNATE AFTER BATCHWISE TREATMENT OF FRACTION B (20 MG.) WITH 200 MG. DEAE-C

| Molarity of $NaH_2PO_4$ | | Results* | | |
|---|---|---|---|---|
| pH 7.0 | pH 4.6 | T.P. | T.A. | S.A. |
| 0.1 | | 9 | 20 | 2 |
| | | 7 | 27 | 4 |
| | 0.1 | 6 | 12 | 2 |
| 0.05 | | 5 | 22 | 4 |
| | | 6 | 24 | 4 |
| | | 5 | 26 | 5 |
| | | 6 | 23 | 4 |
| 0.03 | | 4 | 17 | 4 |
| | | 5 | 20 | 4 |
| | | 4 | 15 | 4 |
| | | 4 | 18 | 5 |
| | | 4 | 15 | 4 |
| | | 5 | 13 | 3 |
| | | 4 | 13 | 3 |
| | | 4 | 20 | 5 |
| | | 4 | 19 | 5 |
| 0.01 | | 2 | 6 | 3 |
| | 0.01 | 1 | 0 | 0 |
| 0.001 | | 0 | 0 | 0 |
| | 0.001 | 0 | 0 | 0 |

* T.P. = total protein (mg.); T.A. = total activity, rat units (R.U.); S.A. = specific activity (R.U./mg.).

was washed successively with 50-ml. portions of 80 per cent EtOH, 95 per cent EtOH, absolute EtOH, an equal mixture of absolute EtOH and ether, and finally ether. The filter paper was removed and the grayish-white precipitate was scraped from the paper and collected.

The yield of material from 1 Gm. of fraction B varied between 200 and 250 mg. The specific activity was 6. A variable loss in total recovery of gonadotropic activity occurred at this step. The loss did not occur as a result of the treatment with DEAE-C, but rather because of incomplete precipitation from the alcohol solution.

*Electrophoresis and column chromatography of fractions A, B, and C*

Several preparations of HPG have been studied by means of paper electrophoresis and column chromatography under different conditions. For these studies we have utilized high-potency (postmenopausal) HPG so that the biologic activity could be determined in the various zones or fractions. These studies will be reported elsewhere. For the present it is of some interest to illustrate the changing composition of fractions A, B, and C during the purification procedure developed. Since considerable purification was achieved, it is to be expected that considerable alteration in protein composition of fractions A, B, and C would result.

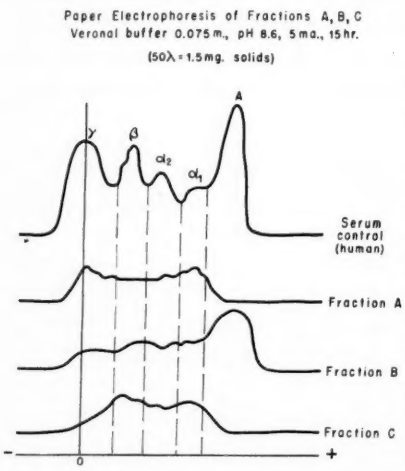



FIG. 1. Electrophoretic patterns of male HPG fractions.

In Figure 1 are shown the results of simultaneous electrophoresis of fractions A, B, and C of HPG from male urine, compared with human serum as a control. In experiments carried out under the same conditions with high-potency material, HPG (as determined by bio-assay) had a mobility in the vicinity of the alpha-1-glycoprotein complex of serum.

In Figure 2 is seen the changing composition of fractions A, B, and C of male HPG as reflected by column chromatography. A rather intensive study of high-potency HPG led us to employ $NH_4Ac$ gradients and mixtures of $NH_4Ac$ and NaCl for elution. For the present studies, 50 mg. of HPG dissolved in 5 ml. of water was placed on a 1.5 by 35-cm. column containing approximately 5 Gm. of DEAE-C. After the hormone solution had penetrated the column, 100 ml. of distilled water was passed through the

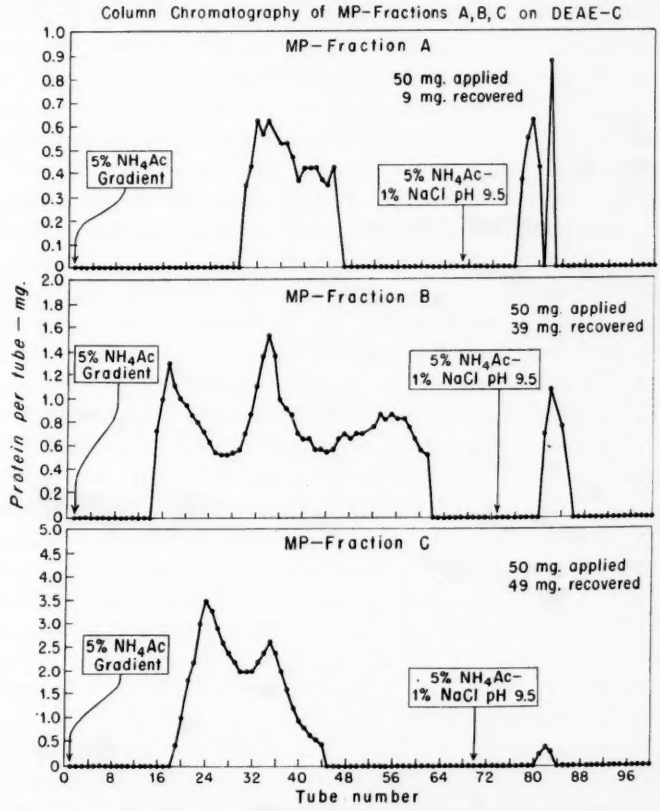



FIG. 2. Column chromatographic patterns of fractions A, B, and C.

column and 100 ml. of effluent was collected. This effluent was devoid of protein and was discarded. A gradient of 150 ml. of 5 per cent $NH_4Ac$ through 150 ml. of distilled water was passed through the column and 4-ml. fractions of effluent were collected. When the gradient was completed, 200 ml. of 5 per cent $NH_4Ac$ solution containing 1 per cent NaCl at a pH of 9.5 was run through the column.

*Biologic activity of fractions A, B, and C*

The number of gonadotropins present in human urine is still a controversial subject. Some investigators claim that HPG is a mixture of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and partial separation of these two hormones has been claimed (see under Comment). This is the commonly accepted view, but it must be borne in mind that it is possible for HPG to exist as a single molecule possessing the biologic prop-

erties of both FSH and LH. Obviously, this problem is of crucial importance in the purification of HPG. All the work reported herein was performed according to our routine method of assay (ovarian-weight method in intact rats). Were HPG composed of two hormones, and had a partial separation of these principles occurred during purification procedures, our routine method of assay would probably not have enabled us to detect such a separation unless it was of major proportion. However, an indication of any separation can probably be obtained by performing fingerprint assays (5). A fingerprint assay consists of determining simultaneously the response of the uterus and the ovary of intact rats to a series of doses of HPG administered at short intervals. The shapes of the curves obtained and certain parameters of these curves (slope and dose ratios) characterize HPG. If the characteristic curve for HPG depends on a certain ratio of FSH to LH, a great alteration in this ratio brought about by separation of FSH from LH during processing would in all probability alter these curves. In Figures 3 to 8 are depicted 6 such fingerprint curves obtained from fractions A, B, and C. Two preparations of fraction A, two preparations of fraction B, and one of C were studied. It is seen that the shapes of all 6 curves are approximately the same. A summary of the parameters of these curves (Table 7) indicates that, by this method, fractions A, B, and C are identical in biologic nature. Probably the most important parameter is the dose ratio (ovary to uterus) at threshold doses. These ratios, which range from 1.8 to 2.2, are similar to those obtained for HPG of men, postmenopausal women, and castrated women (6).

The biologic activity of fractions A, B, and C was assayed by three different methods (Table 8). When assayed by our routine ovarian-weight method on Sprague-Dawley rats bred locally, the potency ratios for fractions A, B, and C were 1, 20, and 80, respectively. When assayed by the augmentation method and the ventral-prostate method (with Sprague-Dawley rats obtained from Madison, Wisconsin), the potency ratios were approximately the same.

The details of the augmentation assay and the results are shown in Figure 9. Three doses of each fraction alone (without chorionic gonadotropin)

TABLE 7. SUMMARY OF PARAMETERS OF FINGERPRINT CURVES*

| Preparation | Liters | Total mg. | Mg./L. | Uterus | | | | Ovary | | | | Dose ratio (O./U.) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | I. at control L. | b | s | λ | I. at control L. | b | s | λ | |
| MP(97–100)A | 240 | 17,536 | 73.1 | 0.35 | 248 | 16 | 0.07 | 0.68 | 93 | 13 | 0.14 | 2.0 |
| MP(97–100)A | | | | 0.31 | 245 | 33 | 0.13 | 0.59 | 100 | 11 | 0.11 | 1.9 |
| MP(101–107)A | 223 | 15,686 | 70.5 | 0.37 | 296 | 22 | 0.07 | 0.67 | 95 | 9 | 0.09 | 1.8 |
| MP(97–100)B | 40 | 136 | 3.4 | 0.30 | 313 | 23 | 0.07 | 0.65 | 99 | 7 | 0.07 | 2.2 |
| MP(91)B | 60 | 294 | 4.9 | 0.28 | 210 | 22 | 0.11 | 0.59 | 120 | 17 | 0.14 | 2.1 |
| MP(97–100)C | 40 | 24 | 0.6 | 0.36 | 245 | 18 | 0.07 | 0.69 | 63 | 10 | 0.16 | 1.9 |

* I. =intercept; L. =liters of urine equivalent; b =slope; s =variance; λ =index of precision; O./U. =ovary/uterus.

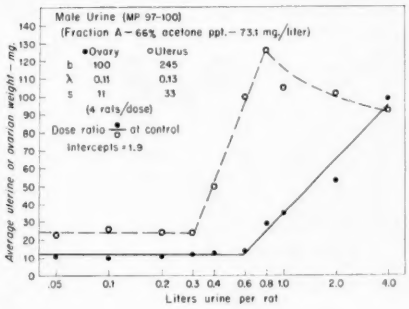

Male Urine (MP 97-100)
(Fraction A – 66% acetone ppt. – 73.1 mg./liter)
•Ovary ○Uterus
b 100 245
λ 0.11 0.13
s 11 33
(4 rats/dose)
Dose ratio •/○ at control
Intercepts = 1.9
Average uterine or ovarian weight – mg.
Liters urine per rat

FIG. 3

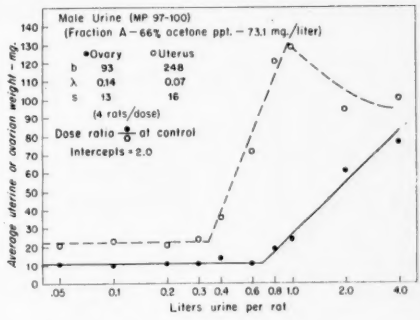

Male Urine (MP 97-100)
(Fraction A – 66% acetone ppt. – 73.1 mg./liter)
•Ovary ○Uterus
b 93 248
λ 0.14 0.07
s 13 16
(4 rats/dose)
Dose ratio •/○ at control
Intercepts = 2.0
Average uterine or ovarian weight – mg.
Liters urine per rat

FIG. 4

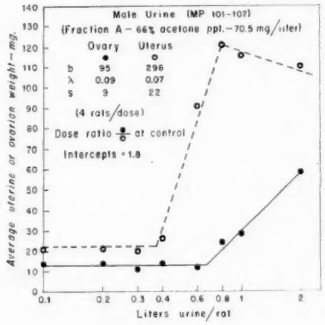



FIG. 5

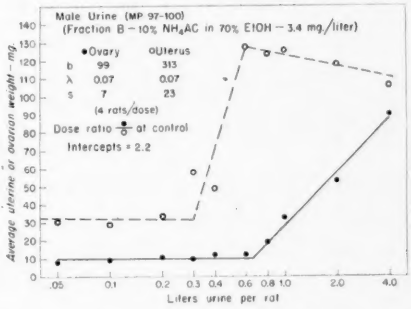

Male Urine (MP 97-100)
(Fraction B – 10% NH4AC in 70% EtOH – 3.4 mg./liter)
•Ovary ○Uterus
b 99 313
λ 0.07 0.07
s 7 23
(4 rats/dose)
Dose ratio •/○ at control
Intercepts = 2.2
Average uterine or ovarian weight – mg.
Liters urine per rat

FIG. 6

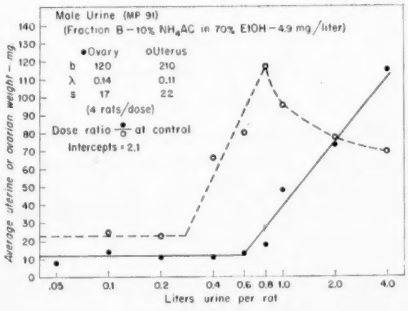

Male Urine (MP 91)
(Fraction B – 10% NH4AC in 70% EtOH – 4.9 mg./liter)
•Ovary ○Uterus
b 120 210
λ 0.14 0.11
s 17 22
(4 rats/dose)
Dose ratio •/○ at control
Intercepts = 2.1
Average uterine or ovarian weight – mg.
Liters urine per rat

FIG. 7

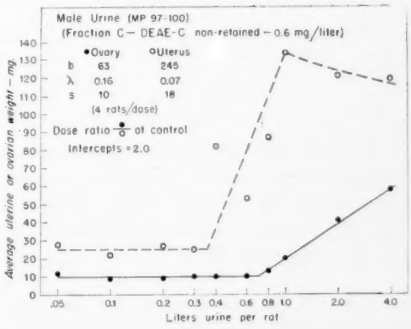
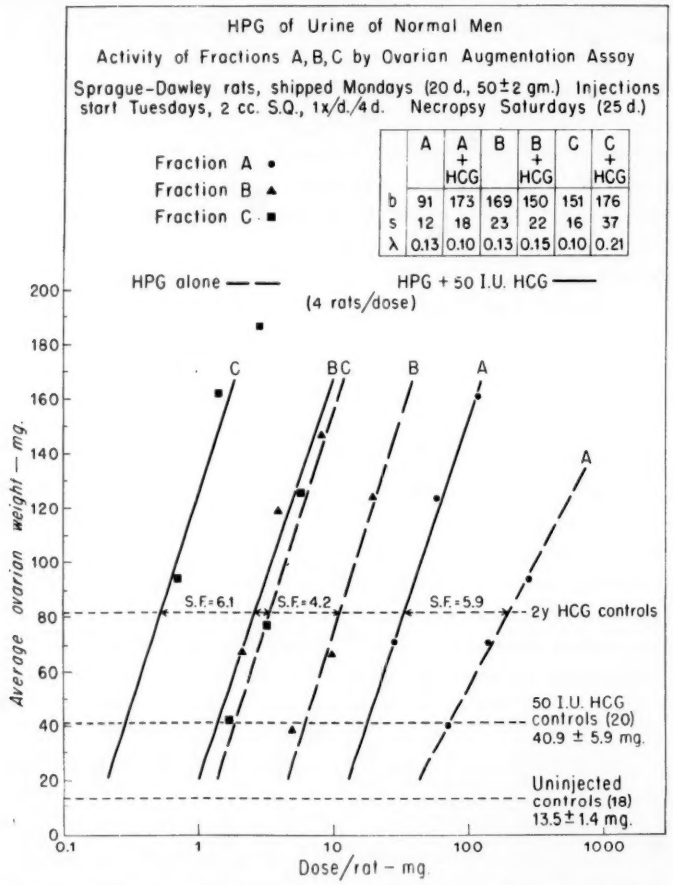

Male Urine (MP 97-100)
(Fraction C – DEAE-C non-retained – 0.6 mg./liter)
•Ovary ○Uterus
b 63 245
λ 0.16 0.07
s 10 18
(4 rats/dose)
Dose ratio •/○ at control
Intercepts = 2.0
Average uterine or ovarian weight – mg.
Liters urine per rat

FIG. 8

**FIGS. 3 to 8. Fingerprint curves of fractions A, B, and C. Fraction C (Fig. 8) and fraction B (Fig. 6) were prepared from fraction A (Fig. 3).**


HPG of Urine of Normal Men
Activity of Fractions A, B, C by Ovarian Augmentation Assay
Sprague-Dawley rats, shipped Mondays (20 d., 50±2 gm.) Injections start Tuesdays, 2 cc. S.Q., 1x/d./4 d. Necropsy Saturdays (25 d.)
Fraction A •
Fraction B ▲
Fraction C ■
HPG alone
HPG + 50 I.U. HCG
(4 rats/dose)
C
BC
B
A
A
S.F.=6.1
S.F.=4.2
S.F.=5.9
2y HCG controls
50 I.U. HCG controls (20) 40.9 ± 5.9 mg.
Uninjected controls (18) 13.5±1.4 mg.
Average ovarian weight — mg.
Dose/rat — mg.

| | A | A + HCG | B | B + HCG | C | C + HCG |
|---|---|---|---|---|---|---|
| b | 91 | 173 | 169 | 150 | 151 | 176 |
| s | 12 | 18 | 23 | 22 | 16 | 37 |
| λ | 0.13 | 0.10 | 0.13 | 0.15 | 0.10 | 0.21 |

FIG. 9. Augmentation assay of fractions A, B, and C. S.F. = sensitivity factor, or increase in sensitivity of response conferred on HPG by HCG, calculated as dose HPG alone divided by dose HPG and HCG at 2y HCG controls. Note also that the slope of fraction A alone is less than that of B and C alone and less than that of 3 fractions in combination with HCG.

were tested. Another set of the same three doses mixed with 50 I.U. of chorionic gonadotropin per rat was simultaneously tested. Two sets of controls (50 I.U. of chorionic gonadotropin alone and uninjected controls) were also included. It was therefore possible to obtain two measures of activity derived from the curves. A unit of ovarian-weight activity of HPG alone is defined as that amount which induces a 100 per cent increase in the ovarian weight over that in the uninjected controls. A unit of ovar-

TABLE 8. ACTIVITY OF FRACTIONS A, B, AND C BY THREE METHODS OF ASSAY*

| Fraction | Routine assay, Sprague-Dawley rats—Rochester | | Assay (Sprague-Dawley rats—Madison) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | O. W. A. | | | O. A. A. | | | V. P. A. | | |
| | R.U./mg. | Pf. | mg./U. | U./mg. | Pf. | mg./U. | U./mg. | Pf. | mg./U. | U./mg. | Pf. |
| A | 0.075 | 1 | 52.0 | 0.019 | 1 | 34.00 | 0.03 | 1 | 43.50 | 0.023 | 1 |
| B | 1.500 | 20 | 5.0 | 0.200 | 11 | 2.60 | 0.40 | 13 | 2.80 | 0.364 | 18 |
| C | 6.000 | 80 | 1.5 | 0.660 | 35 | 0.54 | 1.80 | 60 | 0.98 | 1.020 | 51 |

*R.U./mg. = rat units per mg. = specific activity; Pf. = purification; Mg./U. = amount of HPG needed to produce 100 per cent increase over weight of respective controls; O. W. A. = ovarian-weight activity; O. A. A. = ovarian-augmentation activity; V.P.A. = ventral-prostate activity.

The above assays were performed on material as follows:

Fraction A was prepared from 300 liters of urine. The specific activity was less than (but within the range of) that shown in Table 1. 1 liter urine = 70 mg.

Fraction B was prepared as described by results shown in Table 3. 1 liter urine = 4.8 mg.

Fraction C was prepared from Fraction B. 1 liter urine = 1.1 mg.

ian-augmentation activity is defined as that amount which induces an increase of 100 per cent compared with the weight in the HCG controls. These values are summarized in Table 8.

Finally, fractions A, B, and C were assayed by the ventral-prostate method in hypophysectomized rats obtained from the Hormone Assay Laboratories, Chicago, Illinois; male rats obtained from Sprague-Dawley, Madison, Wisconsin, were used. Details of performance of the assay and the results obtained are given in Figure 10. A unit of ventral-prostate activity was defined as that amount of HPG inducing a 100 per cent increase in the weight of the ventral prostate over that in the uninjected hypophysectomized controls. The purification achieved by the three additional tests was of the same order of magnitude as that obtained with the routine ovarian-weight method.

Because the augmentation assay is supposedly a measure of FSH and the ventral-prostate assay is supposedly a measure of LH, it would appear that no major separation of the two gonadotropins, FSH and LH, had occurred during the purification procedure herein described.

## COMMENT

The literature on the purification of HPG deals chiefly with the HPG of menopausal or postmenopausal women. Only occasional reference is made to male HPG. Segaloff and associates (7) used the Albert method (2) for extracting HPG from postmenopausal urine. The wet 66 per cent acetone precipitate was called "stage 1" material. Stage 1 material was extracted with 10 per cent $NH_4Ac$ in 59 per cent EtOH, and the hormone was precipitated by raising the concentration of alcohol to 80 per cent. The precipitate was referred to as "stage 2" material. All the HPG activity was

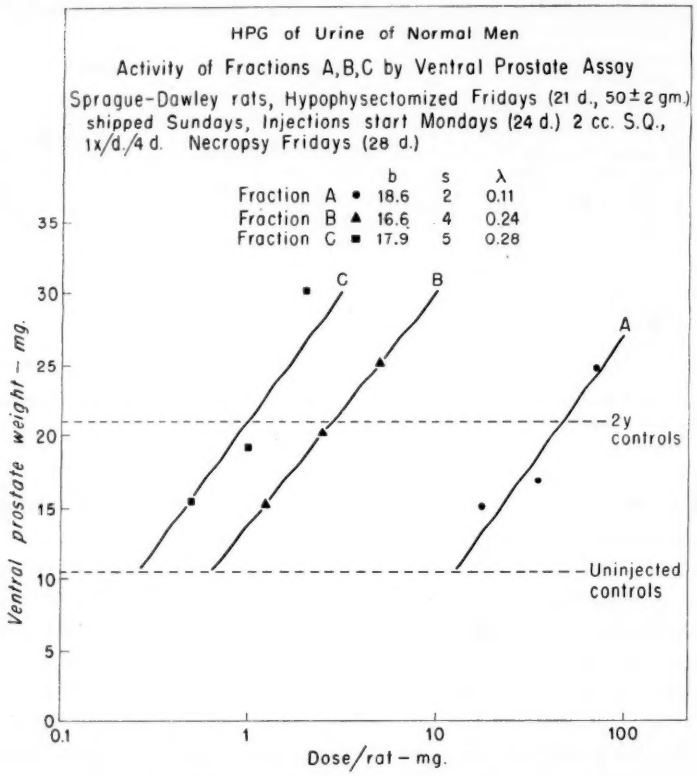

HPG of Urine of Normal Men
Activity of Fractions A,B,C by Ventral Prostate Assay
Sprague-Dawley rats, Hypophysectomized Fridays (21 d., 50±2 gm.) shipped Sundays, Injections start Mondays (24 d.) 2 cc. S.Q., 1x/d./4 d. Necropsy Fridays (28 d.)
b s λ
Fraction A • 18.6 2 0.11
Fraction B ▲ 16.6 4 0.24
Fraction C ■ 17.9 5 0.28
Ventral prostate weight – mg.
Dose/rat – mg.
2y controls
Uninjected controls
A
B
C

FIG. 10. Ventral-prostate assay of fractions A, B, and C.

recovered with one third (Segaloff and Steelman [8]) and two fifths (Segaloff and associates [7]) of the solids originally present in the stage 1 material. This represents a twofold to threefold purification. It was stated that male HPG was similarly prepared (Segaloff and Steelman [8]), but no data were given. With the use of 10 per cent $NH_4Ac$ in 70 per cent EtOH, we have consistently obtained a ninefold to tenfold purification with a 15 per cent loss in total activity from male urine. The difference in results probably is due to the somewhat higher concentration of alcohol used as the extractant, although a difference in the amount of inert material in postmenopausal urine as compared to male urine may also be a factor.

Similarly, little information can be found regarding the behavior of male HPG toward anion exchangers. Segaloff and Steelman (8) applied male HPG (stage 2 material) to a DEAE-C column and eluted the activity with 0.005*M* phosphate buffer at pH 7. The effluent contained LH and was essentially free of FSH. Further elution with a gradient of 0.5*M* NaCl in

0.1*M* $NaH_2PO_4$ removed another fraction, which was chiefly FSH. Thus, a qualitative separation of the HPG complex from male urine was effected.

Anion exchangers had been used with postmenopausal HPG by Butt and Round (9). Apparently one experiment was performed also on male HPG prepared by the kaolin method of Dekanski (10). This HPG preparation would be of low specific activity because the Dekanski method results in extracts containing about 300 mg. of solids per liter of urine. The extract of 2 liters of urine (approximately 600 mg.) was dissolved in 50 ml. of water and treated batchwise twice with DEAE-C. The activity was eluted from the DEAE-C by 0.5*M* $NH_4Ac$ containing 0.2*M* NaCl at pH 9.5. All of the HPG present in the crude material was eluted from the DEAE-C, but it is difficult to determine the purification achieved. The $ED_{50}$ (mouse uterine assay) of the crude material was stated to be less than 3.5, whereas that of the material after treatment with DEAE-C was 1.4. In another paper, Butt and associates (11) subjected male urine to extraction by the benzoic acid-tungstic acid method. A solution of the extract was demineralized and then treated batchwise with DEAE-C, eluted with the above-mentioned $NH_4Ac$-NaCl mixture at pH 9.5, and precipitated by the addition of alcohol. The dried material was dissolved in water and readsorbed on DEAE-C. This time the DEAE-C was treated stepwise, first with 0.2*M* $NH_4Ac$ and then with 0.5*M* $NH_4Ac$ containing 0.2*M* NaCl at pH 9.5. Again, this was apparently a single experiment. Eighteen per cent of the activity initially present was found in the first eluate, and 46 per cent of the activity was present in the eluate of pH 9.5. The specific activity of the first eluate was six times, and that of the second eluate was twice, that of the starting source. Butt and co-workers (11) also studied the behavior of male HPG placed on columns of DEAE-C. The general procedure was to apply HPG to columns in 0.01*M* $NH_4Ac$ and to elute the material by a stepwise increase in concentration with 0.1*M* $NH_4Ac$, 0.2*M* $NH_4Ac$, 0.5*M* $NH_4Ac$, and finally 0.5*M* $NH_4Ac$ containing 0.2*M* NaCl at pH 9.5. Active material was obtained only in the second and fourth elutions, the material in the former being predominantly FSH and in the latter interstitial cell-stimulating hormone (ICSH). Again, a qualitative separation of male HPG into LH and FSH fractions was claimed. It is interesting that the behavior of the two fractions obtained by Butt and associates (11) is opposite to that of the two fractions of Segaloff and Steelman (8). Segaloff and Steelman's LH fraction is less firmly bound than their FSH fraction; the LH of Butt and associates is more firmly bound than their FSH fraction. This may be the result of the different conditions of the chromatography.

The work described herein was proceeding simultaneously without knowledge of the work of Butt and co-workers (11). It is, therefore, of some interest that generally similar procedures and similar eluants were evolved

but that in the end the direction of our efforts was exactly opposite to that of the Butt group. We achieved considerable purification by adsorption of nongonadotropic material to DEAE-C rather than by adsorption of HPG to the anion exchanger. The relative merits of the opposite approaches may be ascertained by comparing the potencies of the material at comparable stages. Butt and associates (11) stated that their male HPG is twice as potent as the standard HMG-20A and " . . . this exceeds the potency of any other preparation from a similar source yet described." Our fraction C is ten to fifteen times more potent than HMG-20A.

As far as any separation of gonadotropins is concerned, it has not been our aim at this stage of the work to effect separation. Rather it has been our intent to remove as much inert material as possible in order to obtain clean preparations of gonadotropin. The basis for this intent is that if a complete separation of FSH and LH of HPG is possible by chemical or physical means, the success of such an accomplishment will be enhanced if purified and concentrated solutions of gonadotropins rather than impure and crude concentrates are used. The correctness of this assumption, however, remains to be proved.

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# INHERITANCE OF DEFECTIVE ORGANIFICATION OF IODINE IN FAMILIAL GOITROUS CRETINISM*

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ABSTRACT

Defective organification of iodine was studied in goitrous cretins and their siblings. The genealogy is presented of 90 members of four generations of two related sibships in which 7 of 11 children in one sibship and 2 of 7 children in the other sibship were cretins. Rapid thyroidal uptake and release of $I^{131}$, prompt and marked discharge of $I^{131}$ after administration of KSCN, and the chromatographic finding of iodide alone in serum and urine up to forty-eight hours after administration of 400 $\mu$c. of $I^{131}$ constituted evidence for a defect in thyroidal organification of iodine in these cretins. Five apparently normal siblings and 8 other relatives studied failed to show any evidence of this defect. Apparently the only mechanism of inheritance that could explain the pattern of this abnormality involves a gene that is completely recessive—at least by present methods of testing.

DEFECTIVE organification of iodine has been studied in goitrous cretins and their siblings (1–3). This report concerns two related sibships of cretins in which $I^{131}$ studies yielded findings compatible with defective thyroidal organification of iodine in the cretins but not in their siblings or other relatives.

## SUBJECTS AND METHODS

### *Subjects*

Figure 1 shows the genealogy of 90 members of four generations of the two sibships under consideration.[1] Seven of 11 children in sibship J. and 2 of 7 children in sibship V. were cretins. The case report of each cretin is presented in the Appendix. A medical history was obtained and a physical examination made on 27 persons, including all the cretins, by one of the authors (R.H.P.) or by a competent private physician. The 4 oldest cretins of sibship J. (Ra., Ri., J. and P.) had each undergone surgical thyroidectomy for nontoxic goiter. In 3 of of these 4, thyroidal $I^{131}$ uptake tests had been performed prior to the thyroidectomy. The surgical pathology of each thyroid gland was reviewed by A. J. French, M.D. (Chairman, Department of Pathology, University of

Received June 9, 1960.

* Aided by Grant M-2399 from the U.S.P.H.S. and Project 15 from the Atomic Energy Commission.

[1] We are grateful to Mrs. Ruth T. Davidson, genetic field worker, and J. V. Neel, M.D., Chairman, Department of Human Genetics, for their aid in this study.

Michigan Medical Center) and L. T. Woolner, M.D. (Pathologist, Mayo Clinic) at our request. Previous medical histories, results of previous physical examinations and laboratory studies were obtained from five hospitals in which all sibship J. cretins had been treated, and from the private physician[2] of sibship V.

*Laboratory tests*

In Table 1 are listed the pertinent data on each cretin in both sibships. In Table 2 are listed the pertinent data on the normal relatives of the cretins in these two sibships. Desiccated thyroid was withheld for six weeks before drawing blood for determination of the serum protein-bound iodine (PBI) (4), butanol-extractable iodine (BEI) (5), and cholesterol levels (6). Thyroid medication was similarly withheld before performing thyroidal $I^{131}$ uptake tests (7), KSCN tests (8), and serum and urine chromatography determinations (9, 10) in cretins more than 6 years of age. In cretins less than 6 years of age, thyroid medication was continued but a dose of 10 units of thyroid-stimulating hormone (TSH)[3] was administered subcutaneously twenty-four hours before the tracer dose of iodine was administered. A 400-$\mu$c. dose of $I^{131}$ was administered as a tracer, for serum chromatography. Blood samples were drawn at two, four and twenty-four hours or at four, twenty-four and forty-eight hours. Urine was collected in twenty-four hour specimens. Both butanol-acetic acid (BAc) and n-butanol-dioxane-ammonia (BAD) solvents were used in descending chromatography. The KSCN tests were usually performed as follows: Conventional determinations were made of the percentage of a dose of $I^{131}$ present in the thyroid gland at one and two hours after oral administration to a subject in a fasting state. An oral dose of 1 Gm. of KSCN in a 25 per cent solution of cherry syrup was then administered. The percentage of the tracer dose of $I^{131}$ present in the thyroid gland was rechecked at 15-minute intervals for one hour, and once again at two hours after administration of KSCN.

## RESULTS

Figure 1 shows the occurrence of goiter and cretinism in the genealogy of the two sibships.

The J. sibship is on the left and the related V. sibship is on the right. Mrs. R.J. was goitrous but not hypothyroid. She gave birth to 11 children[4], 7 of whom were cretins. In the older 5 of these 7 cretins, goiters developed. Four of the 5 were thyroidectomized.

A normal brother (B.C.) of Mrs. R.J. married a woman (D.C.) without thyroid abnormality, and one of their daughters (V.V.) gave birth to the second sibship. This sibship consisted of 7 children; 2 of them were cretins in whom goiter had not developed. The relationship of the two cretinous sibships was that of first cousins once removed.

In Table 1 and Appendix, additional pertinent data are presented on all cretins in both sibships. Although Patient M.V. was not a classic cretin, the serum PBI level of less than 1 $\mu$g. per 100 ml. at 5 weeks of age and the thyroidal 24-hour $I^{131}$ uptake of 3.2 per cent constituted good evidence of

[2] Irving Levitt, M.D.

[3] Thytropar, Armour.

[4] The fetus of a twelfth pregnancy, shown in the table by a small diamond, was lost before term.

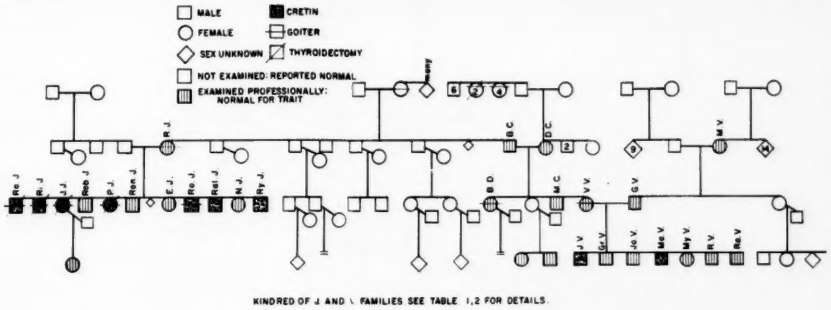


FIG. 1. Pedigree of the J. and V. sibships. The goiters in Patients V.V. and B.D. had been found seven years prior to our examination by an internist elsewhere. In Patient V.V. the use of Lugol's solution apparently resulted in regression of the thyroid to normal size.

markedly subnormal thyroid function since birth.

In Figure 2 are representative thyroidal $I^{131}$ uptake curves for 2 cretins in each sibship.[5] These curves show the rapid early uptake of $I^{131}$ and spontaneous release of $I^{131}$ to low levels at twenty-four hours, as usually found in small thyroid remnants with a rapid turnover of $I^{131}$ and in thyroids with defective iodination of tyrosine.

In Figure 3 these thyroidal $I^{131}$ uptake curves are contrasted with normal curves, *i.e.*, those for the 3 available normal parents of the two sibships plus 2 normal siblings of the J. family cretins.

Evidence for the belief that the rapid uptake and release of $I^{131}$ by the cretins (Fig. 2) is related to defective organic binding and not merely to a normally functioning tiny thyroid remnant is presented in Figure 4 (A and B). This figure shows that, after administration of KSCN, there was a prompt and considerable discharge of $I^{131}$ from the thyroid glands of the 2

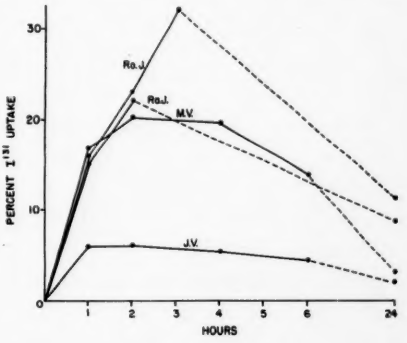


FIG. 2. Thyroidal $I^{131}$ uptakes (per cent of dose) in 2 cretins from each sibship. These curves show the usual rapid early uptake and spontaneous release of $I^{131}$, resulting in a low thyroidal $I^{131}$ content within twenty-four hours after administration of the tracer dose.

[5] In this and in subsequent studies the oldest 2 cretins of the J. sibship who had not been thyroidectomized were the subjects of our investigation.

TABLE 1. PERTINENT DATA ON ALL CRETINS IN BOTH J. AND V. SIBSHIPS

| Subject | Age when seen by us (yrs.) | History | Serum | | | Thyroidal $I^{131}$ uptake (% dose) | | Results of KSCN test | Chromatography | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | PBI ($\mu$g./100 ml.) | BEI ($\mu$g./100 ml.) | Cholesterol (mg./100 ml.) | Early | Late | | Serum | Urine |
| Ra. J. | 23 | Classic cretin at age 3 months. Treatment sporadic. I.Q. 52 at age 12 yrs. *Goiter* at age 17; thyroidectomy. | | | 364 | | | | | |
| Ri. J. | 21 | Classic cretin at 2 months. Treatment sporadic. I.Q. 55 at age 6½ yrs. *Goiter* at age 17; thyroidectomy. | | | | high* at 2 hrs. | low at 33 hrs. | | | |
| J. J. | 19 | Classic cretin at 3 months. *Goiter* at age 15 yrs.; thyroidectomy. I.Q. 45. Treatment sporadic. Apparently had normal offspring at age 21. | | | 416 | high* at 2 hrs. | low at 5 hrs. | | | |
| P. J. | 15 | Classic cretin at 2 months. *Goiter* at age 11 yrs.; thyroidectomy. I.Q. 50 at age 15. Treatment sporadic. | | | 424 | high* at 3 hrs. | low at 5 hrs. | | | |
| Ro. J. | 10 | Classic cretin at 2 months. *Goiter*. Grossly retarded. Treatment sporadic. | 1.6 | 1.4 | 408 | 28.9 at 2 hrs. | 11.4 at 24 hrs. | positive | $I^-$ only at 24 & 48 hrs. | |
| Ral. J. | 9 | Classic cretin at 3–4 months. Grossly retarded. No goiter. Scintiscan, 2 hrs. after $I^{131}$, showed a thyroid gland. Treatment sporadic. | 0.9 | 0.8 | 416 | 22.6 at 2 hrs. | 8.7 at 24 hrs. | positive | largely $I^-$ at 24 & 48 hrs. | |
| Ry. J. | 5 | Classic cretin. Grossly retarded. Treatment sporadic. No goiter | | | | | | | | |
| J. V. | 8 | Classic cretin at 4 months, with no proximal tibial epiphyses and small distal femoral epiphyses. Normal development thereafter, with adequate replacement therapy. Scintiscan showed small thyroid gland. | | | | 6.3 at 2 hrs. | 2.1 at 24 hrs. | positive | $I^-$ only at 4 & 24 hrs. | $I^-$ only at 4 & 24 hrs. |
| M. V. | 3 | Normal appearance at 6 weeks, but low PBI level. Thereafter given adequate replacement therapy; development normal; thyroid not palpable. | less than 1.0 at 6 wks. | | | 20.2 at 2 hrs. | 3.2 at 24 hrs. | positive | $I^-$ only at 4 & 24 hrs. | $I^-$ only at 4 & 24 hrs. |

* $I^{131}$ uptake test performed elsewhere.

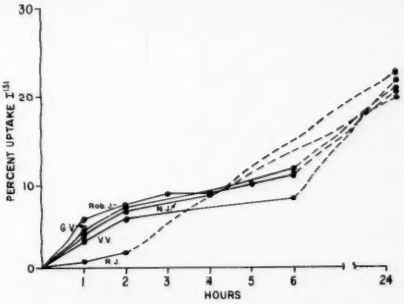


FIG. 3. Normal 24-hour thyroidal $I^{131}$ uptakes in the normal relatives of the cretins in Figure 2.

cretins in the J. sibship (Fig. 4A) and the 2 cretins in the V. sibship (Fig. 4B), characteristic of thyroids with a defect in ability to organify iodine.

Figure 5 shows the contrasting lack of discharge of $I^{131}$ after administration of KSCN in 13 normal close relatives of the cretinous subjects. Additional data on the normal relatives of the cretinous subjects of the two sibships are presented in Table 2. In these apparently normal relatives of the cretins, there was no evidence to suggest that they had the thyroid defect manifested by the cretins.

In order to obtain further evidence regarding the question of whether or not the supernormal release of $I^{131}$ from the thyroid gland of the cretins within twenty-four hours was related to a defect in organic binding, chromatographic analysis was carried out on the serum of 2 of the cretins (R.J. and J.V.). In the V. family, serum and urine chromatography (both BAc and BAD solvents) failed to detect the presence of any $I^{131}$-labeled compound. In the J. family pair of cretins, only a trace of an indefinitely

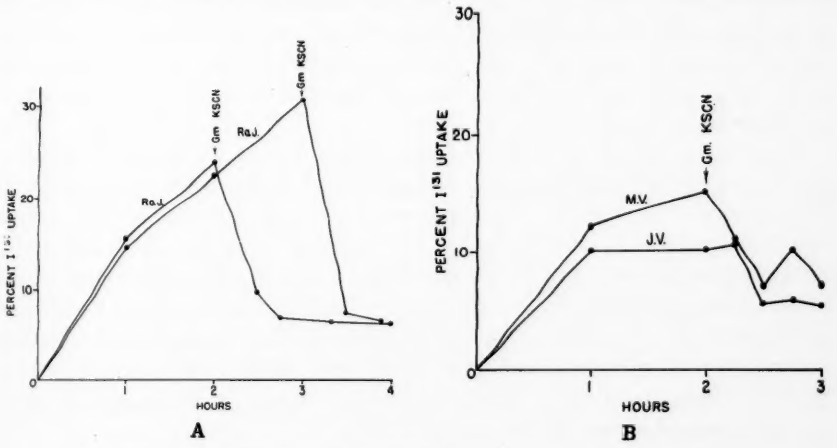


FIG. 4. Rapid discharge of thyroidal $I^{131}$ following administration of KSCN in cretins: **A**) J. sibship; **B**) V. sibship.

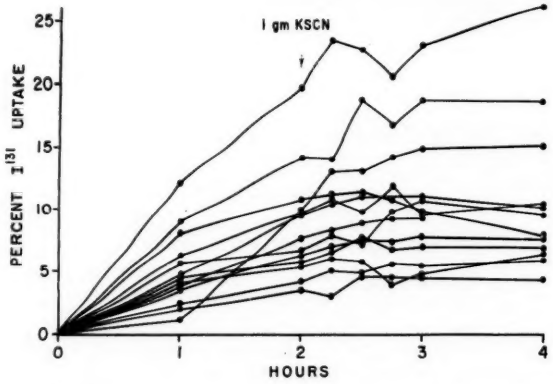


FIG. 5. Normal lack of response of thyroidal $I^{131}$ to administration of KSCN in 13 close relatives of the cretins.

identified $I^{131}$-labeled compound was found; in BAD solvent, the Rf was close to that of triiodothyronine, and in BAc solvent it was close to that of diiodotyrosine.

## DISCUSSION

It is worthy of emphasis that all of the cretins in these two sibships (except the youngest in the J. family who was not available for detailed laboratory study) were shown to have a thyroid gland, as demonstrated by either an unquestionable goiter, a rapid thyroidal uptake of $I^{131}$, or a scintiscan (11) at two hours showing a thyroid pattern in the neck. The scintiscan demonstration of thyroid tissue was of particular importance in the case of Patient J.V. Otherwise, the maximum thyroidal $I^{131}$ uptake of 6.3 per cent, the unrevealing scintiscan at twenty-four hours, and the chromatographic evidence that only iodide was present in the blood and urine suggested that he was athyrotic. In fact, his case previously had been so diagnosed.

The difference in the social and economic situations of the two families led to striking differences in the therapy of the cretinous offspring. The J. family cretins received late and sporadic treatment whereas the V. family cretins were treated almost ideally. Thyroidal $I^{131}$ uptake was lower in the continuously treated pair of cretins (Figs. 2 and 4). This difference could be attributed to long-term suppression of TSH with a consequent decrease in thyroid mass, in contrast to the situation in their inadequately treated goitrous cousins. Such a sequence of events might lead to difficulty in distinguishing "goitrous" from "athyrotic" types, particularly in adequately treated older cretins.

The same economic factors which led to different programs of therapy in these two sibships also resulted in different places of residence and different

TABLE 2. PERTINENT DATA ON NORMAL RELATIVES OF CRETINS IN J. AND V. FAMILIES

| Subject | Age | Relation to cretin | Status | Serum | | | Thyroidal $I^{131}$ uptake (% dose) | | Results of KSCN test | Thyroid auto-antibodies |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | PBI (μg./100 ml.) | BEI (μg./100 ml.) | Cholesterol (mg./100 ml.) | 2 hrs. | 24 hrs. | | |
| R. J. | 43 yrs. | Mother | Examined. Obese with nodular goiter. | 8.6 | | 206 | 9.9 | 22.6 | Neg. | None |
| Rob. J. | 17 yrs. | Sibling | Examined. Normal | | | | | 20.5 | | |
| Ron. J. | 14 yrs. | Sibling | Examined. Normal | 6.0 | 4.4 | | 3.5 | | Neg. | |
| E. J. | 13 yrs. | Sibling | Examined. Normal | 7.2 | 6.0 | 170 | 9.5 | 34.8 | Neg. | |
| N. J. | 8 yrs. | Sibling | Examined. Normal | | | | 5.3 | 19.3 | Neg. | |
| X. J. | 2 mos. | Offspring | Examined in neonatal period by pediatrician. Apparently normal. Roentgenogram of distal femur showed normal epiphysial development. Serum alk. phosphatase, 12 KA units at 2 days | 10.5 (cord blood) 12.0 at age 2 days | | | | | | |
| V. V. | 26 yrs. | Mother | Examined. Normal. History of goiter | 9.1 (pregnant) | | 220 | 5.6 | 21.9 | Neg. | None |
| Go. V. | 28 yrs. | Father | Examined. Normal | | | | 4.2 | 20.5 | Neg. | |
| Gr. V. | 6 yrs. | Sibling | Examined. Normal | | | | 10.7 | | Neg. | |
| Ja. V. | 4 yrs. | Sibling | Examined. Normal | | | | 14.2 | | Neg. | |
| My. V. | 2 yrs. | Sibling | Examined by private pediatrician. Normal | | | | | | | |
| R. V. | 1 yr. | Sibling | Examined by private pediatrician. Normal | | | | | | | |
| Ra. V. | 3 mos. | Sibling | Normal development at 4 mos. Cord serum alk. phosphatase, 7.5 KA units | 9.1 (cord blood) | | | | | | |
| B. D. | 31 yrs. | Aunt | Small palpable goiter. Otherwise normal | | | | 9.8 | | Neg. | |
| M. C. | 12 yrs. | Uncle | Examined. Normal | | | | 6.9 | | Neg. | |
| C. N. | | Cousin | Examined by private pediatrician. Normal | | | | | | | |
| X. N. | 6 mos. | Cousin | Examined by private pediatrician. Normal | 7.7 (cord blood) | 6.7 (cord blood) | | | | | |
| B. C. | 55 yrs. | Grandfather | Examined. Normal | 6.1 | 4.4 | | 19.7 | | Neg. | |
| D. C. | 50 yrs. | Grandmother | Examined. Hypertension; obesity; no goiter | | | | 7.6 | | Neg. | |
| M. V. | 56 yrs. | Grandmother | Examined. Normal | | | | 6.3 | | Neg. | |

social levels, so that exposure to a common goitrogenic agent in their environment is unlikely.

The presence of goiter or demonstrable thyroid tissue in all the cretinous siblings studied, the initial rapid thyroidal uptake of $I^{131}$ and its early release, the prompt discharge of $I^{131}$ from the thyroid after administration of KSCN, and the lack of identifiable compounds other than iodide in the blood or urine up to forty-eight hours after administration of $I^{131}$ are all characteristic of the familial goitrous cretinism described by Stanbury and Hedge (1). The defect in synthesis of thyroid hormone in this type of cretinism occurs at the level of organification of the trapped iodine, presumably because of lack of an oxidative enzyme to aid in the conversion of iodide to iodine. The contrasting lack of these abnormalities in the apparently normal siblings and other relatives of such cretins indicates that there is no similar defect of lesser severity in the noncretinous subjects. This situation differs from that observed in familial goitrous cretinism associated with lack of deiodinating enzyme, in which the "normal," supposedly heterozygous relatives of the cretins may manifest a partial defect, suggesting transmission of the defect by an incompletely recessive gene (12).

That we are dealing with an inherited defect in the present study appears unquestionable, since there is no other plausible explanation for the occurrence of a rare identical defect in close sibships. The exact mode of inheritance remains uncertain. Ordinarily it would be unlikely for a rare trait transmitted by a single recessive gene to appear in two branches of a family in which no consanguinity could be traced, since this mode of inheritance would require heterozygous mothers of both sets of cretins to have married a heterozygous carrier of the same rare recessive gene. On the other hand, such an occurrence is not impossible, and some of the characteristics of recessive inheritance were present: 1) the familial nature of the disease, with the parents of the cretins being normal in appearance; 2) the suggestive ratio of affected to unaffected of approximately 1 to 3, particularly in the V. family; and 3) the birth of apparently normal offspring to an affected person.

The known presence of goiter in the mothers of each sibship of cretins and 8 other members of the genealogy covering four generations suggests the possibility that in the heterozygous state there may be a minor defect in organification of iodine, undetectable by our methods. Therefore, although the inheritance of this defect is through an autosomal recessive gene, we cannot be certain that it is completely recessive. It is unlikely that the mothers had Hashimoto's struma, since they had no demonstrable circulating thyroid auto-antibodies (13, 14) and there was no discharge of thyroidal $I^{131}$ after administration of KSCN.

The evidence suggests that this is an inherited defect. The mode of in-

heritance is apparently by transmission of the trait through a gene that is completely recessive, as judged by present methods of testing. The presence of goiter in otherwise normal relatives of such cretins requires further clarification.

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## Appendix

*J. Sibship*

*Ra. J.* (male) weighed 8 pounds 4 ounces at birth. The clinical diagnosis of cretinism was made at 3 months of age. His I.Q. was 52 at the age of 12 years, and his bone age was 9 years at the chronologic age of 14 years. He underwent thyroidectomy at age 17 for a goiter. No carcinoma was found; the thyroid tissue showed the usual changes associated with goitrous cretinism, according to Drs. Woolner and French.

*Ri. J.* (male) weighed 11 pounds ¾ ounce at birth. The clinical diagnosis of cretinism was made at 2 months of age. His bone age was retarded 3½ years at the chronologic age of 10 years. His I.Q. was 55 at age 6½ years. A thyroidectomy performed at age 17, for a goiter; the thyroid tissue was morphologically similar to that in Patient Ra. J.

*J. J.* (female) weighed 11 pounds at birth. The diagnosis of cretinism was made at 3 months of age. The bone age was retarded 4½ years at the chronologic age of 8 years. The I.Q. was 45. Surgical thyroidectomy at age 15, for a goiter, yielded tissue similar to that found in her siblings. At age 21, she gave birth to a normal infant.

*P. J.* (female) weighed 10 pounds 13 ounces at birth. The diagnosis of cretinism was made at 2 months of age. The bone age was retarded 1 year at chronologic age 10. She was mute. A thyroidectomy at age 11, for a goiter, yielded tissue similar to that found in her siblings. Her I.Q. was 50 at age 15.

*Ro. J.* (male) weighed 8 pounds 7½ ounces at birth. The diagnosis of cretinism was made at 2 months of age when he was hospitalized because of choking and cyanosis produced by obstruction due to his large tongue. Mental retardation was severe, and he was excluded from school. On examination at age 10 years, he had a palpable thyroid gland with a nodule (2–3 cm.) in the isthmus.

*Ral. J.* (male) weighed 11 pounds 12 ounces at birth. The diagnosis of cretinism was made at 4 months of age. At the time of examination, he was an idiot with poor bladder control. A goiter was not detectable clinically, but a scintiscan showed the presence of a thyroid.

*Ry. J.* (male) weighed 8 pounds 7½ ounces at birth. On examination at 5 years of age, he had the clinical appearance of a cretin without goiter. Mental retardation was severe, and there was no bladder control.

*Parents*: *Mrs. J.*, the mother of the foregoing 7 cretins, was normal on examination except for obesity and a small nodular goiter. *Mr. J.*, the father of this family, was reputed to be normal and to work regularly, but he was not available.

*V. Sibship*

*J. V.* (male) weighed 7 pounds 6 ounces at birth and was found to be a classic cretin at 4 months of age. He was treated regularly with adequate amounts of thyroid, and subsequent development was normal. He had only minor difficulty in keeping up with his regular class in school. After six weeks without thyroid medication at age 8, he became lethargic and gained weight.

*M. V.* (male) weighed 8 pounds 13 ounces at birth. Because of a cretinous sibling, a serum PBI determination was made at 6 weeks of age, although the patient appeared normal; the result was reported as less than 1.0 μg. per 100 ml. The 24-hour thyroidal $I^{131}$ uptake was 13 per cent. Adequate thyroid therapy was given continuously thereafter and development was normal. There were no stigmata of cretinism. The thyroid was not palpable.

*Parents. Mr. and Mrs. V.*, mother and father of this pair of cretins, were normal physically and mentally. The mother had been found to have a goiter by an internist seven years prior to the time we saw her. Her goiter had apparently disappeared when she was treated with Lugol's solution of iodine. A pronounced history of goiter was present on the mother's side of this family. Six sisters of her maternal grandfather had a goiter; in 2, it was large enough to require thyroidectomy. The maternal grandfather of this family, who was the brother of the mother of the J. family, was entirely normal. He demonstrated no thyroidal defect after administration of KSCN. His serum PBI level was 6.1 μg., and BEI level 4.4 μg. per 100 ml., with less than 0.5 μg. of inorganic iodide per 100 ml. The maternal and paternal grandmothers of this family were normal women and did not exhibit any thyroidal defect after administration of KSCN.

# SOME EFFECTS OF MAGNESIUM LOADING IN PATIENTS WITH THYROID DISORDERS*

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ABSTRACT

The effects of 1.0 Gm. of $Mg^{++}$ administered intravenously twice daily were studied in 1 euthyroid patient, 2 patients with primary myxedema and 2 patients with hyperthyroidism. There was no change in the basal metabolic rate in either the euthyroid or the hyperthyroid patients; magnesium given for three days following an injection of triiodothyronine ($T_3$) in the hypothyroid patients did not block the calorigenic effect of $T_3$. No consistent alterations of the urinary excretion of nitrogen or phosphorus were noted and the phosphaturic effect of $T_3$ was not inhibited by magnesium. Variable amounts of magnesium were retained by each patient. In all 5 patients, a marked increase in urinary calcium excretion followed infusion of magnesium; in 4 patients, the serum calcium level was normal or low, whereas in the fifth patient it was elevated. These studies do not appear to indicate any consistent antagonistic effect of magnesium on the peripheral functions of thyroid hormone in man; further studies are necessary for clarification of the relationship between magnesium and calcium.

IT IS well recognized that magnesium has an important role in the functions of muscle and nerve tissues (1) and in the actions of many enzyme systems (2). Magnesium interferes with the action of thyroxine in uncoupling oxidative phosphorylation *in vitro* (3, 4). In hyperthyroidism, an increased concentration of serum protein-bound magnesium has been reported (5, 6); however, there has not been uniform agreement regarding this observation (7, 8). Administration of triiodothyronine to patients with myxedema results in a significant rise in the urinary excretion of magnesium (9). For these reasons, it was considered worthwhile to investigate further the relationship between thyroid function and magnesium metabolism in man and to ascertain whether the administration of magnesium affects the peripheral action of the thyroid hormones.

Received May 21, 1960.

* Supported in part by Research Grant A-1459, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland.

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## CLINICAL MATERIAL AND METHODS

Studies were performed on 1 euthyroid patient who had just recovered from lobar pneumonia, 2 patients with primary myxedema and 2 patients with hyperthyroidism. All were females; their ages ranged from 24 to 57 years. The clinical diagnoses of thyroid function were substantiated by determinations of the basal metabolic rate (BMR), serum protein-bound iodine level (PBI) , and thyroidal $I^{131}$ uptake. The patients received diets constant in protein, fat and carbohydrate for six-day periods. The basal metabolic rate was determined in duplicate before breakfast by the same technician using a clinical closed-circuit apparatus. Daily 24-hour collections of urine were preserved with 10 ml. of glacial acetic acid, and were analyzed for content of nitrogen (10), magnesium (11), calcium (12) and phosphorus (13). Serum concentrations of magnesium, calcium, phosphorus and PBI[1] were determined on blood samples taken from patients in the fasting state, except as noted in the tables.

Magnesium was administered intravenously as magnesium sulfate equivalent to 1.0 Gm. of $Mg^{++}$ in 300 ml. of 5 per cent dextrose, over a period three hours twice daily, morning and evening , for three successive days. Administration was monitored by careful observation of the deep tendon reflexes, respiratory rate and blood pressure; calcium gluconate was available for immediate intravenous use, but at no time was it needed, although both hypothyroid patients complained of weakness following the infusions of magnesium. *l*-Triiodothyronine[2] was dissolved in a few drops of 0.01 *N* NaOH, diluted with physiologic salt solution, autoclaved and administered to the patients with myxedema as a single dose of 1.0 mg. subcutaneously, except for one occasion when 0.1 mg. was given intravenously to 1 patient.

## RESULTS

In the euthyroid woman (L.M., Table 1) the administration of magnesium did not alter appreciably the BMR level or the serum PBI concentration. Values for the concentration of serum magnesium obtained one hour after cessation of the morning infusions of magnesium on two occasions were significantly above the normal range. This patient excreted in the urine about 50 per cent of the magnesium administered. Urinary nitrogen on the first two days of magnesium administration was less than on the preceding control days, but on the third experimental day rose to a level which was subsequently maintained following the infusions of magnesium. Urinary excretion of phosphate increased slightly on the second and third days of magnesium administration, but concentrations of serum phosphorus were relatively constant. Urinary excretion of calcium was much greater on the days of magnesium infusions than on control days; concentrations of serum calcium were in the low normal range.

In the first patient with hypothyroidism (M.C., Table 2), the initial injection of triiodothyronine ($T_3$) induced in the following three days an

[1] Determinations of serum protein-bound iodine concentration were performed by Bio-Science Laboratories, Los Angeles, California.

[2] *l*-Triiodothyronine was supplied through the courtesy of Dr. A. E. Heming of Smith, Kline and French Laboratories.

elevation of the BMR, no significant changes in the level of serum PBI or urinary excretion of calcium, and a slight increase in urinary nitrogen. There was, however, a marked rise in urinary excretion of magnesium and phosphorus. The second injection of $T_3$ was followed by infusions of magnesium for three days. The BMR again rose, although not to as great an extent as after the first dose of triiodothyronine. The serum concentration of magnesium was significantly elevated one hour after an infusion of magnesium; urinary excretion of magnesium rose, but still accounted for only about 50 per cent of the magnesium administered. Urinary nitrogen remained fairly constant and urinary phosphorus did not increase to the same extent as after the first dose of $T_3$. On the other hand, urinary calcium

TABLE 1. METABOLIC EFFECTS OF ADMINISTRATION OF MAGNESIUM IN A 24-YEAR-OLD EUTHYROID FEMALE (L.M.)

| | Day* | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| BMR (%) | −13 | −5 | −1 | | −5 | 0 | −12 | −3 | +2 | +4 | | −7 | −5 |
| Serum Mg (mg./100 ml.) | 1.7 | 2.7 | | | 2.0 | 2.4 | 6.7† | 3.2 | 3.3 | | | 3.2 | |
| | | | | | | | | 4.7† | | | | | |
| Serum Ca (mg./100 ml.) | 7.7 | 9.0 | | | 8.2 | 7.9 | 8.0† | 7.4 | 9.1 | | | 8.3 | |
| Serum P (mg./100 ml.) | 3.7 | 4.2 | | | 3.8 | 4.1 | 4.2† | 3.7 | 4.3 | | | 3.2 | |
| Serum PBI (μg./100 ml.) | 5.7 | | | | | 4.5 | | 4.9 | 5.4 | | | | |
| Urine Mg. (mg./24 hrs.) | 93 | 105 | 121 | 147 | 121 | 996 | 821 | 1548 | 283 | 90 | 72 | 114 | 98 |
| Urine N (Gm./24 hrs.) | 6.4 | 7.4 | 8.4 | 7.4 | 6.2 | 3.3 | 2.7 | 4.9 | 4.1 | 3.8 | 4.7 | 5.0 | 4.2 |
| Urine P (mg./24 hrs.) | 612 | 525 | 587 | 550 | 695 | 665 | 794 | 915 | 550 | 462 | 424 | 488 | 433 |
| Urine Ca (mg./24 hrs.) | 50 | 53 | 65 | 56 | 95 | 224 | 260 | 199 | 22 | 13 | 29 | 32 | 41 |

* Magnesium administered on days 6, 7, and 8.
† Determined one hour after the end of a magnesium infusion.

increased markedly (as it did in the control patient) and serum concentrations of calcium decreased on the days of the magnesium infusions.

The second patient with hypothyroidism (M.S., Table 3) was given 0.1 mg. of triiodothyronine ($T_3$) intravenously; however, in order to induce a greater metabolic change, a dose of 1.0 mg. of $T_3$ was administered subcutaneously four days later, after which there was a marked rise in BMR. Urinary magnesium and nitrogen increased, but the excretion of calcium and, surprisingly, of phosphorus was not altered appreciably. After an interval of seven days, another subcutaneous injection of 1.0 mg. of $T_3$ was given and this was followed by infusions of magnesium for three days. Again there was a significant rise in BMR, but little change in the level of serum PBI. The serum concentration of magnesium was elevated one hour after an infusion of magnesium was finished and was still above the normal level five hours later. In contrast to the findings in the 2 previous patients, about 80 per cent of the magnesium administered was excreted in the urine. Urinary nitrogen showed a slight rise above the amount excreted before administration of $T_3$ and magnesium. Hypercalciuria and hyperphosphaturia occurred; the serum calcium level declined and the serum phosphorus level rose on the days of the magnesium infusions.

TABLE 2. METABOLIC EFFECTS OF ADMINISTRATION OF TRIIODOTHYRONINE AND MAGNESIUM IN A 44-YEAR-OLD HYPOTHYROID FEMALE (M.C.)

| | Day* | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| BMR (%) | −31 | −22 | −27 | −30 | −34 | −6 | −5 | −2 | −3 | −13 | −22 | −10 | −8 | −9 | −11 |
| Serum Mg (mg./100 ml.) | 2.4 | 2.3 | | | 2.6 | | 2.9 | | 2.2 | | 2.9 | 6.2† | 4.1 | | 3.5 |
| Serum Ca (mg./100 ml.) | 7.8 | 7.6 | | | 8.5 | | 8.8 | | 8.6 | | 8.6 | 6.5† | 6.1 | | 8.0 |
| Serum P (mg./100 ml.) | 3.6 | 3.3 | | | 3.1 | | 3.3 | | 3.4 | | 3.2 | 3.9† | 2.8 | | 3.0 |
| Serum PBI (μg./100 ml.) | 2.1 | | | | 2.0 | | 2.6 | | 2.4 | | 2.3 | 3.0† | 3.1 | | 1.1 |
| Urine Mg (mg./24 hrs.) | 37 | 27 | 21 | 20 | 56 | 137 | 115 | 86 | 53 | 79 | 804 | 1795 | 1298 | 151 | 66 |
| Urine N (Gm./24 hrs.) | 9.9 | 7.5 | 9.1 | 6.7 | 7.5 | 11.2 | 10.6 | 10.1 | 9.0 | 9.7 | 10.0 | 8.6 | 9.1 | 9.9 | 10.1 |
| Urine P (mg./24 hrs.) | 682 | 595 | 538 | 420 | 1035 | 1482 | 768 | 603 | 601 | 558 | 456 | 880 | 505 | 452 | 427 |
| Urine Ca (mg./24 hrs.) | 89 | 65 | 56 | 65 | 55 | 94 | 66 | 48 | 32 | 40 | 380 | 326 | 256 | 40 | 45 |

* Magnesium administered on days 11, 12 and 13.
Triiodothyronine administered on days 5 and 11.
† Determined one hour after the end of a magnesium infusion.

TABLE 3. METABOLIC EFFECTS OF ADMINISTRATION OF TRIIODOTHYRONINE AND MAGNESIUM IN A 53-YEAR-OLD HYPOTHYROID FEMALE (M.S.)

| | Day* | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
| BMR (%) | −38 | −38 | −38 | −33 | −14 | −4 | | −11 | +9 | +8 | +8 | +8 | −3 | | −25 | +21 | +33 | +25 | +19 | +11 | |
| Serum Mg (mg./100 ml.) | | 3.5 | | 3.5 | | 4.3 | | 3.6 | | 3.6 | | 3.4 | | | 4.2 | 6.9<br>8.8†<br>7.2‡ | 7.1<br>5.7‡ | 7.9 | | 4.3 | |
| Serum Ca (mg./100 ml.) | | 11.1 | | 11.6 | | 10.3 | | 10.6 | | 9.6 | | 9.2 | | | 9.3 | 8.2<br>7.6†<br>7.3‡ | 6.8<br>7.7‡ | 6.6 | | 8.6 | |
| Serum P (mg./100 ml.) | | 4.7 | | 4.2 | | 4.6 | | 4.2 | | 4.8 | | 4.4 | | | 4.8 | 8.6<br>8.4†<br>8.7‡ | 7.3 | 5.4 | | 3.6 | |
| Serum PBI (μg./100 ml.) | 1.2 | | | 1.0 | | 0.5 | | 0.5 | | 0.9 | | 1.3 | | | 0.9 | | 1.3 | 1.5 | | 0.9 | |
| Urine Mg (mg./24 hrs.) | 42 | 61 | 40 | 35 | 43 | 58 | 77 | 45 | 54 | 95 | 115 | 130 | 98 | 101 | 1083 | 2012 | 2211 | 587 | 174 | 107 | 103 |
| Urine N (Gm./24 hrs.) | 4.7 | 7.3 | 5.8 | 7.8 | 6.0 | 6.9 | 8.4 | 8.0 | 11.2 | 12.0 | 12.4 | 12.1 | 12.4 | 14.2 | 11.0 | 13.1 | 15.6 | 15.0 | 12.9 | 12.5 | 11.2 |
| Urine P (mg./24 hrs.) | 497 | 468 | 494 | 738 | 591 | 559 | 505 | 603 | 795 | 650 | 218 | 344 | 552 | 982 | 954 | 660 | 1510 | 1295 | 877 | 824 | 576 |
| Urine Ca (mg./24 hrs.) | 77 | 97 | 52 | 53 | 50 | 64 | 87 | 79 | 76 | 108 | 84 | 64 | 72 | 105 | 337 | 410 | 436 | 119 | 82 | 35 | 26 |

* Magnesium administered on days 15, 16 and 17.
Triiodothyronine administered on days 4, 8 and 15.
† Determined one hour after the end of a magnesium infusion (before lunch).
‡ Determined five hours after the end of a magnesium infusion (before supper).

TABLE 4. METABOLIC EFFECTS OF ADMINISTRATION OF MAGNESIUM IN A 57-YEAR-OLD HYPERTHYROID FEMALE (A.C.)

| | Day* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| BMR (%) | +20 | +30 | +24 | | +4 | +4 | +25 | +27 | +12 | +12 | | +15 |
| Serum Mg (mg./100 ml.) | 2.3 | | | | 2.4 | | 3.8 | 8.4† | 2.4 | 2.3 | | 2.1 |
| Serum Ca (mg./100 ml.) | 11.4 | | | | 11.4 | | 10.4 | 10.2† | 10.6 | 10.4 | | 10.1 |
| Serum P (mg./100 ml.) | 5.7 | | | | 5.2 | | 6.2 | 5.3† | 6.2 | 5.7 | | 5.4 |
| Serum PBI (μg./100 ml.) | 5.9 | 7.0 | | | 7.0 | | | | 6.6 | | | 7.5 |
| Urine Mg. (mg./24 hrs.) | 64 | 109 | 79 | 103 | 94 | 915 | 1857 | 1559 | 226 | 108 | 137 | 90 |
| Urine N (Gm./24 hrs.) | 7.7 | 10.8 | 8.8 | 10.8 | 9.2 | 8.7 | 8.5 | 8.9 | 9.5 | 6.9 | 9.5 | 9.8 |
| Urine P (mg./24 hrs.) | 524 | 746 | 596 | 613 | 638 | 533 | 623 | 766 | 708 | 531 | 726 | 577 |
| Urine Ca (mg./24 hrs.) | 58 | 116 | 66 | 106 | 76 | 227 | 500 | 512 | 260 | 171 | 222 | 222 |

* Magnesium administered on days 6, 7 and 8.
Though the BMR and PBI level were not consistently elevated above normal, this patient had mild symptoms of hyperthyroidism. Thyroidal $I^{131}$ uptake was 75% in 6 hours and 47% in 24 hours; studies with $I^{131}$-thyroxine revealed rapid disappearance from the serum, with a half-life of 4.1 days. One month later the patient returned with the full-blown picture of Graves' disease.
† Determined one hour after the end of a magnesium infusion.

The 2 patients with hyperthyroidism (A.C., Table 4; M.T., Table 5) received infusions of magnesium for three days, preceded and followed by control periods of five days and four days, respectively. In neither patient did the BMR or the serum PBI level change significantly on the days of administration of magnesium. Patient A.C. excreted 66 per cent and Patient M.T. excreted 80 per cent of the magnesium given. The concentration of serum magnesium was elevated above the normal range in both patients one hour after the end of the infusions. Urinary excretion of nitrogen was not altered significantly throughout the periods of study. Hypercalciuria occurred on the days of the magnesium infusions; the serum calcium level remained normal in Patient A.C., but rose in Patient M.T. after the magnesium infusions. Urinary phosphorus did not change in Patient A.C. but increased in Patient M.T. after administration of magnesium; serum phosphorus values were consistently elevated in Patient A.C., but were normal in patient M.T. even following the magnesium infusions.

TABLE 5. METABOLIC EFFECTS OF ADMINISTRATION OF MAGNESIUM IN A 23-YEAR-OLD HYPERTHYROID FEMALE (M.T.)

| | Day* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| BMR (%) | +38 | +37 | | +43 | +38 | +42 | +27 | +43 | +32 | +37 | +47 | +45 |
| Serum Mg (mg./100 ml.) | 2.0 | | | 1.5 | | 2.9<br>5.7† | | 3.2<br>3.0‡ | 2.2 | | 1.7 | |
| Serum Ca (mg./100 ml.) | 9.8 | | | 10.0 | | 8.6<br>13.3† | | 8.8<br>12.8‡ | 10.2 | | 10.6 | |
| Serum P (mg./100 ml.) | 3.8 | | | 3.7 | | 3.8<br>2.5† | | 4.9<br>4.6‡ | 5.6 | | 3.9 | |
| Serum PBI (μg./100 ml.) | 14.1 | | | 12.7 | | | | 15.0 | 14.2 | | | |
| Urine Mg (mg./24 hrs.) | 109 | 163 | 91 | 78 | 72 | 1456 | 1732 | 1973 | 260 | 134 | 124 | 107 |
| Urine N (Gm./24 hrs.) | 8.4 | 7.9 | 5.2 | 4.4 | 4.9 | 8.3 | 6.1 | 7.7 | 8.9 | 10.0 | 6.9 | 5.5 |
| Urine P (mg./24 hrs.) | 575 | 517 | 239 | 261 | 212 | 921 | 466 | 706 | 467 | 406 | 495 | 387 |
| Urine Ca (mg./24 hrs.) | 129 | 141 | 169 | 120 | 116 | 836 | 772 | 812 | 273 | 261 | 315 | 152 |

* Magnesium administered on days 6, 7 and 8.
† Determined one hour after the end of a magnesium infusion (before lunch).
‡ Determined five hours after the end of a magnesium infusion (before supper).

## DISCUSSION

From these limited observations, certain comments seem justified. In preliminary studies, magnesium was administered intramuscularly. However, it was found that in order to attain the desired increments in the concentration of magnesium in the serum, it was necessary to inject such large amounts of magnesium sulfate intramuscularly that the patients refused to tolerate the procedure because of extreme pain. Yet it was difficult to persuade any patient to accept a continuous intravenous infusion for twenty-four hours. Therefore, the magnesium was given intravenously for three hours twice daily, as previously described. Initial trials of this procedure in both normal and hyperthyroid patients revealed that the serum level of magnesium remained elevated for at least three hours following the end of the infusion; thus it could be presumed that the serum magnesium concentration was above normal for at least twelve out of the twenty-four hours on these days. In the patients of the present series (Tables 1–5) the concentration of magnesium in the serum one hour after infusion was significantly elevated. Total serum magnesium levels were normal in the fasting state, both in the patients reported here (except for Patient M.S., with hypothyroidism and a slightly elevated concentration of serum magnesium) and in other patients with hyperthyroidism and hypothyroidism whom we have studied.

The prime purpose of the present studies was to ascertain whether magnesium would block the peripheral action of the thyroid hormones in man, since it has been demonstrated by several investigators that magnesium inhibits the action of thyroxine in uncoupling oxidative phosphorylation *in vitro* (3, 4). Under the conditions of the present studies, administration of magnesium did not affect the basal metabolic rate in either the euthyroid or the hyperthyroid patients. Likewise, in the 2 hypothyroid patients, intravenous administration of magnesium following injection of triiodothyronine did not block the calorigenic effect of the latter. Since injection of $T_3$ usually exerts its maximum effect on the BMR about forty-eight to seventy-two hours later (14), it was thought that administration of magnesium for three days immediately following administration of triiodothyronine would show whether or not magnesium had any effect. However, the serum level of magnesium may not have been elevated constantly during this period and the tissue concentration of magnesium may not have risen to a degree sufficient to cause any peripheral effect on oxygen utilization. It has been reported (15) that the BMR was not altered in 2 subjects by induction of magnesium deficiency. No consistent effects of administration of magnesium were noted on urinary excretion of nitrogen or phosphorus. Although it is well known that administration of $T_3$ results

in increased excretion of urinary nitrogen and phosphorus (14, 16), these actions of $T_3$ were not inhibited by the administration of magnesium.

It has been reported previously (9) that administration of $T_3$ results in increased urinary excretion of magnesium, and this effect was observed in the 2 hypothyroid patients of the present study after the first dose of $T_3$. However, the euthyroid subject, 1 hyperthyroid patient (A.C.) and 1 hypothyroid patient (M.C.) retained a large part of the administered magnesium; the other hyperthyroid patient (M.T.) and the other hypothyroid patient (M.S.) excreted in the urine most of the magnesium which they received. It has been shown (17) that very little of the magnesium given intravenously is excreted in the feces. These results indicate that magnesium balance does not necessarily depend upon the state of thyroid function. In all 5 patients studied here, urinary calcium excretion increased markedly on the days on which magnesium was administered. This effect of magnesium has been noted previously (18). Some of the excess urinary calcium may represent excess sulfate (19).

Although the present studies are brief, they do not appear to indicate any consistent antagonism between magnesium and the peripheral functions of thyroid hormones in man. One report mentions abnormal distribution of $Mg^{28}$ in myxedema (20). Further studies, using newer methods for determination of magnesium and magnesium[28], should provide a better understanding of any relationship between magnesium metabolism and the action of the thyroid hormones.

*Acknowledgment*

The author is grateful for the technical assistance rendered by Mrs. Beatrix Machin in the performance of these studies.

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# CONJUGATED 17-HYDROXYCORTICOSTEROIDS IN PLASMA: MEASUREMENT AND SIGNIFICANCE IN RELATION TO SURGICAL TRAUMA*

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ABSTRACT

Measurement of the concentration of 17-hydroxycorticosteroid (17-OH-CS) glucuronide conjugates in plasma has been suggested as a means of estimating changes in the rate of free steroid metabolism. In the present study a modification of the Nelson-Samuels technique was utilized for the measurement of 17-OH-CS concentration in serum after glucuronidase hydrolysis. This technique proved to be reliable; the results coincided with those obtained by previously reported procedures. The response of the serum free and conjugated 17-OH-CS in normal subjects to maximal adrenal stimulation with corticotropin was compared to the response in patients within seventy-two hours after subtotal gastrectomy. The rise in the serum level of free 17-OH-CS was more rapid following gastrectomy in the surgical patients than following adrenal stimulation in the normal subjects; however, the rise in the serum level of conjugated 17-OH-CS was more gradual in the surgical patients. In view of the variation between subjects, these differences were not statistically significant. In adrenalectomized dogs receiving a constant intravenous infusion of hydrocortisone, laparotomy resulted in a rise in the plasma level of free 17-OH-CS. This manifestation of a delay in steroid metabolism was not correlated with a fall in the plasma level of conjugated 17-OH-CS; the concentration of the conjugates either remained the same or increased. It is concluded that the serum concentration of glucuronide conjugates is determined by multiple factors, only one of which is the rate of their production. As such, their concentration in serum does not reflect the changes in the rate of steroid metabolism that often occur following surgical operations.

Received May 31, 1960

* This work was sponsored by the Subcommittee on Metabolism in Trauma, Advisory Committee on Metabolism, Office of the Surgeon General, Department of the Army, and supported in part by a grant from the United States Atomic Energy Commission and in part by a United States Public Health Service Grant.

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HYDROCORTISONE is the predominant 17-hydroxycorticosteroid in human plasma as measured by the Nelson-Samuels method (1–3). The concentration of hydrocortisone is dependent not only upon the rate of its production by the adrenal cortex, but also upon the rate of its metabolic destruction and excretion (4). This has been emphasized by studies in a variety of clinical conditions in man. Following operations (5, 6), and in association with cirrhosis of the liver (7–9), pregnancy (10, 11) and myxedema (12), a delay in the disappearance rate of exogenous hydrocortisone from plasma has been documented.

Most of the 17-hydroxycorticoids (17-OH-CS) identifiable in urine, by virtue of prior metabolic destruction, appear as water-soluble glucuronide conjugates of the reduction products of hydrocortisone (13–15). These steroid conjugates exist in plasma in significant concentration prior to their excretion by the kidney (16). Although they are generally considered to be biologically inactive, interest has focused on their measurement in blood (17–20) in an attempt to evaluate the influence of changes in intermediary metabolism on the availability of the biologically active, "free" adrenal steroids. It has been suggested that measurement of the total plasma 17-hydroxycorticosteroids (both free and conjugated), could afford an index of adrenal secretory activity which is independent of variation in the rate of hydrocortisone metabolism (17). By inference, this suggestion implies that the concentration of plasma conjugated corticosteroids should afford an index of the rate of metabolic destruction of the adrenal steroids, reflecting a direct relationship to the rate of steroid metabolism.

The present report presents the results obtained from measuring steroid conjugates with an adaptation of the Nelson-Samuels method (21) for the determination of free 17-OH-CS in plasma. In an attempt to evaluate the biologic significance of this measurement as an index of postoperative change in steroid metabolism, the following studies were undertaken:

1. The response of normal subjects to maximum effective infusions of corticotropin (ACTH) was compared to the response in patients undergoing major operations.
2. The effect of operations on the plasma concentration of free and conjugated 17-OH-CS was observed in adrenalectomized dogs receiving a constant infusion of hydrocortisone.

## METHODS

The concentration of free 17-hydroxycorticosteroid was measured by the method of Nelson and Samuels (21) on 10-ml. samples of plasma.

The plasma, after chloroform extraction of the free 17-OH-CS, was brought to pH 5.0 with 50 per cent acetic acid. β-Glucuronidase[1] was added to a final concentration of

[1] Ketodase (beef liver β-glucuronidase), Warner-Chilcott Laboratories.

1000 units per ml, and the mixture was incubated for twenty-four hours at 40°C. The product was subjected to extraction three times with 1.5 volumes of freshly distilled chloroform. The chloroform extracts were combined and evaporated to dryness. The residue was partitioned twice between freshly distilled benzene and water, 2:5, (22). The benzene phase was discarded; the aqueous phase was treated by extraction three times with 1.5 volumes of chloroform. The combined chloroform extracts were filtered through 2 Gm. of anhydrous sodium sulphate into 125-ml. flasks and evaporated to dryness. The residue was taken up in 5 ml. of chloroform for chromatography on Florisil columns. The procedure from this point was exactly as described by Nelson and Samuels for the determination of free 17-hydroxycorticosteroids.

## EVALUATION OF METHOD

### *Specificity*

Absorption spectra of the Porter-Silber (23) chromogens extracted from plasma by the present method coincided with the spectra of pure hydrocortisone and tetrahydrocortisol, with the peak absorption at 410 millimicrons. The uniformly small correction for background was carried out by the Allen formula (24).

### *Reproducibility and recovery*

Consecutive analyses of human and canine serum pools demonstrated that results with the present method are reproducible within approximately $\pm 10$ per cent[2] in normal concentrations.

The mean recovery of hydrocortisone added to duplicate aliquots from a pool of human serum was $93.1 \pm 8.8$ per cent,[2] with a range of 78.0 to 107.0 per cent. Recovery studies following the addition of pure steroid glucuronides were precluded by the unavailability of these substances. Previous studies, however, have demonstrated complete hydrolysis of pure glucuronides by procedures similar to the one just described (19). In addition, neither by prolonging the duration of hydrolysis, nor by increasing the concentration of $\beta$-glucuronidase could the yield be increased over that obtained by the present procedure.

## BIOLOGIC RESULTS

### A. *Clinical Findings*

### *Normal values*

The mean plasma concentration of conjugated 17-OH-CS in 32 samples of blood drawn between 8:00 and 10:00 a.m. from 16 normal subjects was $5.0 \pm 4.8$ $\mu$g. per 100 ml., with a range of 0.0 to 18.1 $\mu$g. The mean plasma concentration of free 17-OH-CS in the same samples was $11.2 \pm 5.2$ $\mu$g. per 100 ml. These normal values are comparable to those reported by other investigators (18, 25, 26).

### *Response to ACTH*

Nine tests on 5 normal subjects formed the basis for determining the pattern of the response of free and conjugated 17-OH-CS to maximal adrenal stimulation with corticotropin. These tests consisted of the con-

[2] Standard deviation.

tinuous intravenous infusion of 25 units of ACTH over an eight-hour period. Individual results for each subject are presented in Table 1. The mean response is presented in Figure 1. These results coincide with those previously reported by other authors (26–28).

*Response to operations*

In Table 2 and Figure 2 are presented the results of studies on 9 patients at intervals ranging from four to seventy-two hours after the beginning of subtotal gastrectomy. General (ether) anesthesia was used in each case. In none of these patients was there clinical evidence of coincident hepatic or renal disease. The response of the plasma conjugated 17-OH-CS level following operation was similar to that previously reported (27–30).

The rise in the level of free 17-OH-CS was greater following operation than in the normal subjects following maximal stimulation with ACTH. This indicated a delay in the rate of metabolism of the free steroids following operation. A delay in the rate of conjugation following operation was also suggested, since the rate of increase in the concentration of conjugates was not so rapid postoperatively as in normal subjects following the administration of ACTH. The differences in the response of the free and conjugated steroids to ACTH and operation are of doubtful significance, however, in view of the variability between patients. Similar variability has been previously noted (27).

B. *Findings in Animal Experiments*

*Preparation*

Seven dogs were studied. Four days prior to the experimental "run," an inlying sampling catheter was placed in the left central hepatic vein (31). A bilateral adrenalectomy was performed, the spleen was removed, and a portal vein catheter was introduced through the splenic vein. A similar sampling catheter was placed in the splenic artery in 5 of the animals. The animals were studied according to two separate procotols.

*Procotols*

In 5 dogs (Nos. 1, 4, 5, 7 and 9—Table 3, Fig. 3) a constant infusion of hydrocortisone was administered through a hind-limb vein at a rate approximating 5 mg. per Kg. per twenty-four hours. After four hours and at hourly intervals thereafter, samples of blood were collected from each catheter. Eight hours after the beginning of the infusion, each animal was anesthetized with pentobarbital and an exploratory laparotomy performed. The operative procedures each lasted one hour. Blood samples were withdrawn at the completion of the operation and at hourly intervals for an additional five hours. Each 20-ml. sample was immediately replaced by an

TABLE 1. RESPONSE TO MAXIMAL ADRENAL STIMULATION WITH ACTH

Free and conjugated 17-OH-CS levels in plasma of normal subjects during an eight-hour intravenous infusion of 25 units of ACTH (0–8 hours).

| Subject | Plasma 17-hydroxycorticosteroids (μg./100 ml.) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 hour | | 4 hours | | 6 hours | | 8 hours | | 10 hours | |
| | Free | Conj. | Free | Conj. | Free | Conj. | Free | Conj. | Free | Conj. |
| BU | 12.4 | 7.4 | 32.4 | 8.8 | 42.2 | 22.9 | 42.9 | 22.2 | | |
| MU | 6.6 | 17.3 | 23.4 | 28.8 | 26.0 | 40.0 | 29.7 | 51.3 | 24.3 | 23.1 |
| JA | 1.9 | 0.0 | 25.3 | 16.7 | 23.0 | 19.3 | 30.4 | 22.2 | 7.4 | 15.0 |
| JA | 9.4 | 13.3 | 30.6 | 28.0 | 27.2 | 32.7 | 29.4 | 38.7 | 12.8 | 33.3 |
| JA | 17.7 | 7.9 | 25.7 | 17.0 | 21.7 | 17.0 | 27.3 | 23.5 | 16.8 | 23.6 |
| BE | 9.9 | 6.5 | 20.4 | 15.6 | 18.0 | 16.7 | 24.1 | 16.7 | 6.8 | 17.5 |
| BE | 3.8 | 4.6 | 17.2 | 13.3 | 20.8 | 18.5 | 18.4 | 28.3 | 5.4 | 26.0 |
| LI | 25.5 | 2.2 | 63.5 | 12.2 | 69.2 | 19.5 | 78.4 | 17.2 | 46.1 | 14.3 |
| LI | 12.5 | 18.1 | 22.2 | 21.2 | 51.1 | 35.2 | 65.4 | 41.9 | 69.6 | 51.4 |

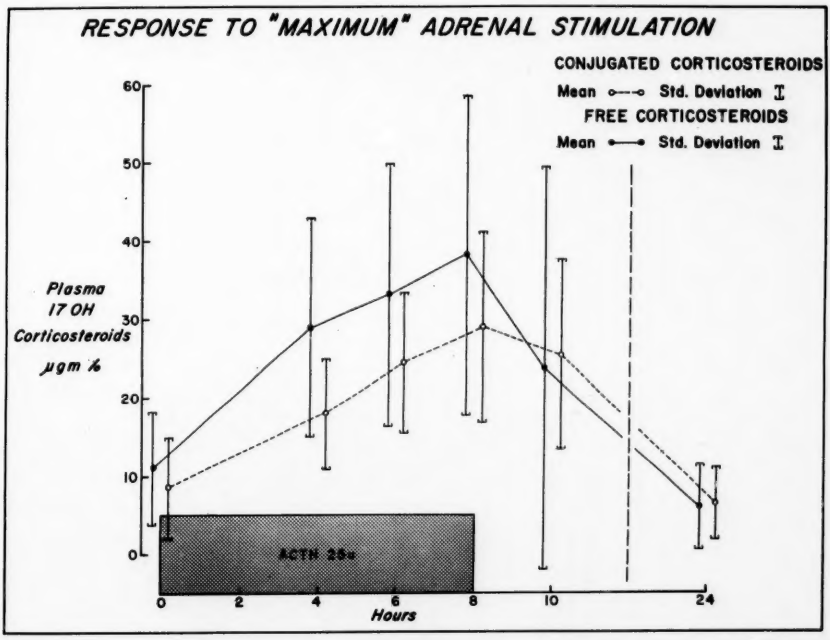


FIG. 1. Response of plasma free and conjugated 17-OH-CS to the intravenous infusion of 25 units of ACTH over an eight-hour period. Values are the mean concentrations and standard deviations for 9 individual "tests" on 5 normal subjects.

TABLE 2. RESPONSE TO OPERATION (GASTRECTOMY)

Free and conjugated 17-OH-CS concentrations in plasma of patients during and after subtotal gastrectomy (operation approximately 0–4 hours in each instance).

| Patient | Plasma 17-hydroxycorticosteroids (μg./100 ml.) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 hour* | | 4 hours** | | 6 hours | | 8 hours | | 10 hours | | 24 hours | | 48 hours | | 72 hours | |
| | Free | Conj. | Free | Conj. | Free | Conj. | Free | Conj. | Free | Conj. | Free | Conj. | Free | Conj. | Free | Conj. |
| TA | 11.0 | 6.5 | 38.1 | 20.2 | 43.7 | 27.1 | 46.6 | 23.1 | 43.9 | 33.7 | 22.9 | 23.6 | | 14.6 | 11.6 | 9.8 |
| GL | 16.3 | 1.2 | 36.8 | 5.5 | 39.9 | 13.5 | 54.1 | 33.3 | 53.7 | 38.9 | 23.8 | 8.0 | 15.9 | 1.6 | 21.3 | 16.7 |
| GO | 5.5 | 0.0 | 41.6 | 19.2 | 36.9 | 17.8 | 41.3 | 29.2 | 31.2 | 33.5 | 33.8 | 22.7 | | | 28.2 | 10.0 |
| CA | 14.6 | 0.0 | 30.0 | 16.7 | 34.3 | 24.1 | 41.1 | 42.7 | 46.8 | 53.6 | 28.6 | 66.7 | 30.6 | 24.1 | 20.3 | 21.3 |
| RO | 3.1 | 0.0 | 29.4 | 8.0 | 32.3 | 15.7 | 41.7 | 20.8 | 46.9 | 25.2 | 23.4 | 12.4 | 33.9 | 15.6 | | |
| MA | 16.7 | 5.9 | 40.2 | 9.8 | 27.4 | 14.0 | 43.9 | 10.3 | 31.5 | 14.4 | 20.0 | 11.8 | 18.6 | 10.6 | 15.4 | 5.9 |
| RA | 11.3 | 0.0 | | 6.3 | 40.6 | 6.9 | 44.5 | 23.8 | 44.2 | 28.5 | 41.0 | 22.7 | 41.3 | 25.0 | 29.6 | 12.5 |
| McK | 3.8 | 4.4 | 47.8 | 14.9 | 48.5 | 11.5 | 37.5 | 14.6 | 34.8 | 10.5 | 25.3 | 9.3 | 21.9 | 6.8 | 17.0 | 6.0 |
| WA | 8.7 | 2.7 | 38.3 | 20.8 | 47.5 | 20.2 | 49.7 | 34.5 | 76.2 | 16.3 | 32.0 | 21.8 | | 14.3 | 18.0 | 7.6 |

* At beginning of operation.
** At end of operation.

equal volume of fresh whole canine blood. The heparinized samples were centrifuged and the plasma frozen for subsequent analysis.

In an additional 2 dogs (Nos. 3 and 10—Table 4, Fig. 4), a constant infusion of hydrocortisone was administered through a hind-limb vein. After eight hours the infusion site was changed from the hind-limb vein to the previously catheterized portal vein. Sampling was the same as in the dogs undergoing laparotomy, and was continued for six hours after the change of infusion site. The infusion rate in terms of μg. per hour in each animal in both groups was calculated from the observed infusion rate (ml. per hour) and quadruplicate analyses of the infusate.

*Results*

*Site of conjugation.* In the course of these experiments, 31 paired samples of blood were obtained from the portal and hepatic veins in adrenalectomized dogs receiving hydrocortisone by constant peripheral venous infusion. These dogs were unanesthetized and in as normal a state as could be

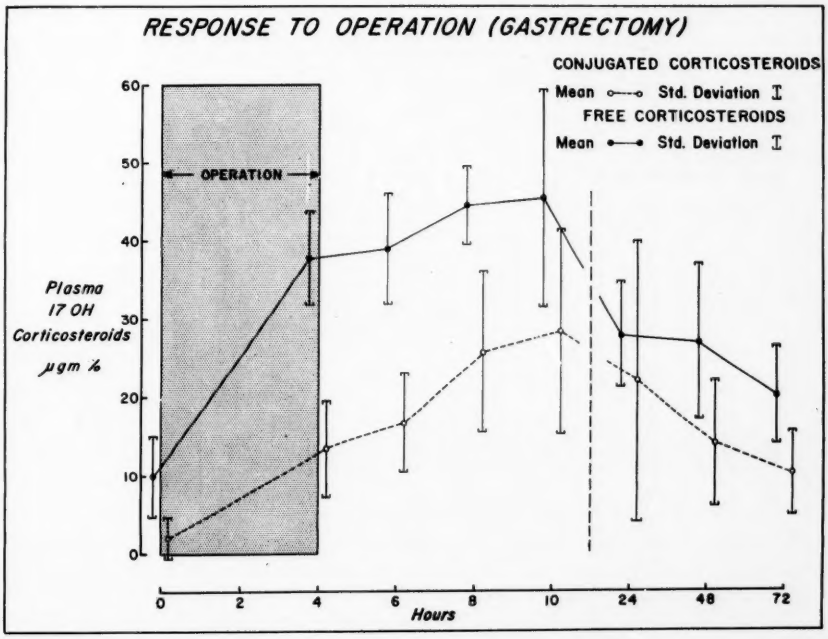


FIG. 2. Response of plasma free and conjugated 17-OH-CS to subtotal gastrectomy. Values are the means and standard deviations. Sampling times were the same in all patients. The four-hour samples were obtained near the end, or shortly after the operation in each instance.

TABLE 3. EFFECT OF LAPAROTOMY ON CORTICOSTEROID METABOLISM IN ADRENALECTOMIZED DOGS

Mean concentrations and standard deviations of free and conjugated 17-OH-CS in portal venous (PV), splenic arterial (SA), and hepatic venous (HV) plasma of adrenalectomized dogs receiving a constant infusion of hydrocortisone. After four hours of infusion, plasma concentrations had reached a steady state. Preoperative samples of blood were then obtained at hourly intervals for four hours prior to exploratory laparotomy (which lasted one hour). Samples were also obtained at the end of the operation and at hourly intervals postoperatively.

| Dog No. | Plasma 17-hydroxycorticosteroids (μg./100 ml.) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preoperative | | | | | | Postoperative | | | | | |
| | Conjugated | | | Free | | | Conjugated | | | Free | | |
| | PV | SA | HV | PV | HV | PV-HV | PV | SA | HV | PV | HV | PV-HV |
| 1 | 11.6 ± 2.9 | | 9.1 ± 7.7 | 32.6 ± 5.0 | 23.1 ± 4.2 | 9.5 ± 2.5 | 14.0 ± 4.2 | | 14.0 ± 0.5 | 36.9 ± 5.5 | 26.6 ± 2.1 | 10.4 ± 5.9 |
| 4 | 9.0 ± 2.3 | | 8.2 ± 5.0 | 16.4 ± 2.6 | 10.0 ± 2.6 | 6.5 ± 2.4 | 7.8 ± 6.3 | | 8.3 ± 4.7 | 21.1 ± 3.3 | 12.4 ± 2.8 | 8.7 ± 3.7 |
| 5 | 6.0 ± 2.9 | 5.2 ± 3.3 | 3.3 ± 3.0 | 23.2 ± 1.0 | 14.3 ± 3.0 | 8.6 ± 2.1 | 6.0 ± 1.6 | 5.5 ± 3.0 | 6.3 ± 1.3 | 26.6 ± 1.7 | 19.7 ± 3.2 | 6.6 ± 4.0 |
| 7 | 7.6 ± 1.9 | 7.0 ± 3.0 | 8.6 ± 4.5 | 37.0 ± 4.8 | 18.1 ± 2.2 | 18.8 ± 3.6 | 10.7 ± 1.4 | 12.7 ± 1.5 | 14.6 ± 4.8 | 64.5 ± 7.6 | 33.0 ± 10.4 | 31.5 ± 12.1 |
| 9 | 5.5 ± 0.9 | 6.3 ± 1.8 | 4.8 ± 4.2 | 14.6 ± 2.0 | 5.8 ± 2.6 | 8.3 ± 3.8 | 8.0 ± 3.8 | 5.6 ± 1.8 | 5.0 ± 4.3 | 18.4 ± 4.6 | 11.4 ± 3.0 | 8.6 ± 4.4 |

achieved. When the concentrations of conjugated 17-OH-CS in portal and hepatic venous plasma, respectively, were treated as a ratio (PV/HV), the resultant mean for the series was $1.44 \pm 1.44$ (S.D.). Twenty-one paired samples of blood were obtained under the same conditions from the portal vein and splenic artery. The mean ratio (PV/SA) of the concentrations of conjugated 17-OH-CS for this series was $1.22 \pm 0.97$. In the 2 dogs in which the constant infusion of hydrocortisone was given through the portal vein, 10 paired samples of blood were obtained from the splenic artery and hepatic vein, respectively. The ratio (SA/HV) of the concentrations of conjugated 17-OH-CS for this series was $1.49 \pm 2.0$.

Thus we could detect no significant arterio-venous difference of conjugated 17-OH-CS in the splanchnic circulation of the adrenalectomized

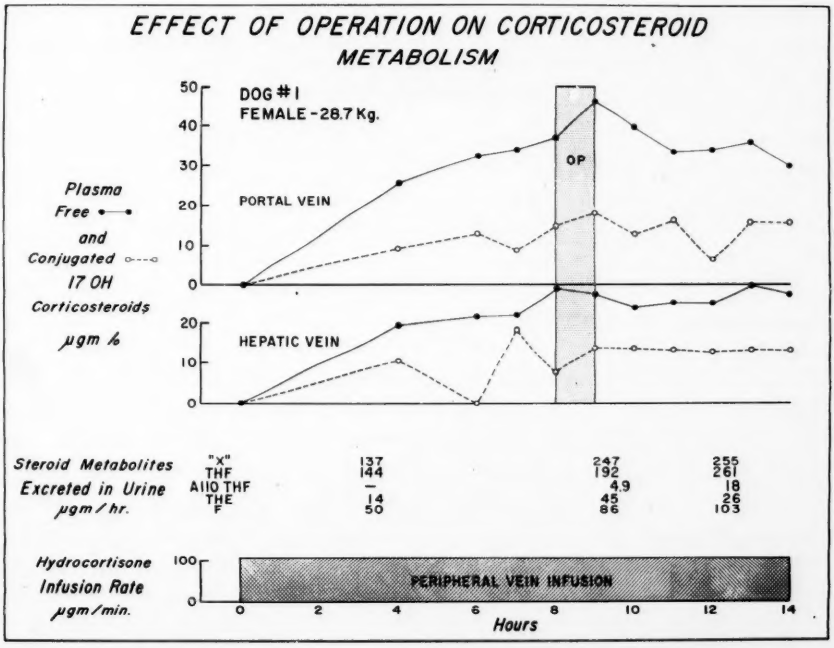


FIG. 3. Effect of an exploratory laparotomy on the plasma free and conjugated 17-OH-CS levels in an adrenalectomized dog receiving a constant infusion of hydrocortisone. Plasma values for blood obtained from the portal vein and the hepatic veins are presented separately. Also shown are the urinary excretion rates of the Porter-Silber metabolites of hydrocortisone (x = an unidentified substance; THF = tetrahydrocortisol; Allo THF = allo tetrahydrocortisol; THF = tetrahydrocortisone; and F = hydrocortisone). The samples of urine excreted during various periods of the experiment were pooled for chromatographic separation as indicated (0 to 8 hours, 8 to 11 hours, and 11 to 14 hours).

TABLE 4. PERIPHERAL VERSUS PORTAL VENOUS INFUSION OF HYDROCORTISONE

Mean concentrations and standard deviations of free and conjugated 17-OH-CS in portal venous (PV), splenic arterial (SA), and hepatic venous (HV) plasma of dogs receiving a constant infusion of hydrocortisone. After four hours of peripheral venous infusion, plasma concentrations had reached a steady state and samples were then obtained at hourly intervals At eight hours the infusion site was changed from a peripheral vein to the portal vein, and sampling was continued at hourly intervals.

| Dog No. | Plasma 17-hydroxycorticosteroids (μg./100 ml.) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Peripheral vein infusion | | | | | | | Portal vein infusion | | | |
| | Conjugates | | | Free | | | | Conjugates | | Free | |
| | PV | SA | HV | PV | SA | HV | $\frac{PV+SA}{2}-HV$ | SA | HV | SA | HV |
| 3 | 9.2± 3.7 | 5.5± 1.9 | 8.1± 2.8 | 22.0± 1.8 | 19.6± 1.2 | 8.8± 2.6 | 12.0± 1.9 | 7.1± 1.5 | 6.8± 2.3 | 12.8± 1.7 | 13.6± 2.1 |
| 10 | 3.0± 4.1 | 3.0± 3.5 | 3.7± 5.1 | 7.1± 1.7 | 6.8± 1.7 | 3.7± 1.0 | 3.3± 1.7 | 4.8± 4.4 | 2.4± 2.9 | 7.7± 3.1 | 8.3± 3.1 |

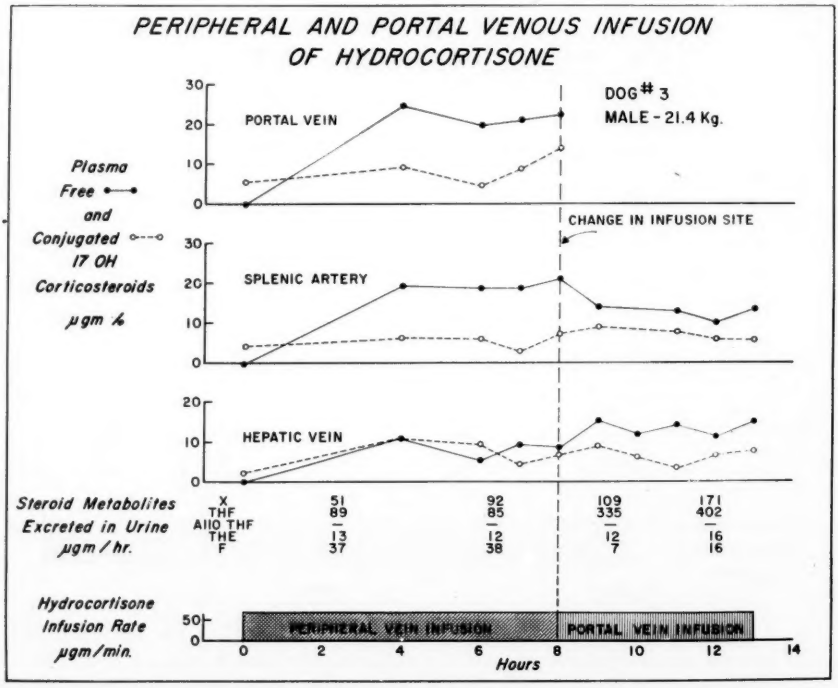


FIG. 4. The effect of altering the site of administration of a constant infusion of hydrocortisone from a leg vein to the portal vein in an adrenalectomized dog. Plasma values for free and conjugated 17-OH-CS in blood samples obtained from the portal vein, splenic artery and hepatic veins are presented separately. Also shown are the urinary excretion rates of Porter-Silber metabolites of hydrocortisone during individual periods of the experiment as indicated (0 to 4 hours, 4 to 8 hours, 8 to 11 hours, and 11 to 13 hours).

dog receiving a constant peripheral venous infusion of hydrocortisone. Similarly, no hepatic gradient of conjugated 17-OH-CS was discernible during either the peripheral venous or portal venous infusion.

In 2 dogs (Nos. 1 and 3—Figs. 3 and 4), quantitative determinations were made of the excretion rates of the Porter-Silber metabolites of hydrocortisone in urine.[3] The maximum total excretion rates of these substances were 635 and 573 μg. per hour, respectively. At normal rates of hepatic blood flow (33) the production of this quantity of metabolites solely by the liver would result in hepatic gradients of 17-OH-CS (2 μg. per 100 ml. or less) which could not be reliably measured by the present method.

*Effect of operation.* In the adrenalectomized animals studied before and after laparotomy, the effect of trauma on the concentration of plasma conjugated 17-OH-CS was evaluated (Fig. 3, Table 3). The plasma concentrations of both conjugated and free 17-OH-CS were virtually stable before laparotomy after four hours of constant infusion of hydrocortisone. Following operation, there was a consistent rise in the concentration of free 17-OH-CS in all samples. Apparently there was a postoperative delay in the metabolism of hydrocortisone. No coincident fall in the concentration of conjugated 17-OH-CS was observed; this value either remained unchanged or rose slightly after laparotomy. There was no correlation between the postoperative delayed disappearance of free 17-OH-CS and the diminution in the rate of production of the conjugated 17-OH-CS as reflected by the serum concentration of conjugates.

## DISCUSSION

From 20 to 30 per cent of hydrocortisone injected into a normal human subject is eventually recoverable in the urine as 17-hydroxycorticosteroids (34). Of this fraction, only a minor proportion is directly extractable with chloroform, and thus represents "free" steroid (13). The remainder can be extracted only after hydrolysis with β-glucuronidase, and presumably represents glucuronide conjugate. This latter fraction is composed in large part of tetrahydrocortisol, tetrahydrocortisone, and allotetrahydrocortisol (35). The empirical concept that compounds which are not reduced in ring A are not conjugated, and that excretion products which are found to be conjugated have been reduced, suggests that ring A reduction is a prerequisite for the occurrence of conjugation. Destruction of hydrocortisone is nearly abolished by hepatectomy in the experimental animal (36). It appears, then, that the liver is the major site of 17-OH-CS metabolism. Indeed, specific enzymes which catalyze the reduction of cortisol have been isolated from hepatic tissue (37, 38). The actual site of conjugation of the

[3] These data were derived from quantitative paper chromatographic analyses which were kindly performed by Dr. Norman Gold. The procedures used in these determinations have been previously reported (32).

metabolites of the adrenal steroid hormones, however, remains to be conclusively demonstrated.

Hardy and Turner (39) concluded that conjugation occurs rapidly and in considerable proportion in peripheral blood, where the reaction is enhanced by the presence of an "X" substance derived from the liver. Hume and Bell (40), however, were unable to confirm the basic observation that the concentration of conjugated 17-OH-CS is greater in adrenal vein blood than in peripheral blood. In the present studies we were unable to demonstrate a concentration gradient of conjugated 17-OH-CS across the liver. Neither was an arterio-venous gradient discernible.

Comparison of the rise in plasma conjugated 17-OH-CS in the normal subjects receiving maximum ACTH stimulation with that in patients following subtotal gastrectomy indicates that a delay occurred in the appearance of conjugated steroids after operation. Coincidentally, there was a more profound rise in the concentration of free 17-OH-CS in response to surgical trauma in comparison with the response to ACTH in normal subjects. This suggests that there was a delay in conjugation in the postoperative state and that this was reflected by a delay in the rate of metabolism of free 17-OH-CS. However, these results varied sufficiently between subjects to make this conclusion open to question.

Following laparotomy in the adrenalectomized dog receiving a constant infusion of hydrocortisone, there was no decrease in the plasma concentration of conjugated 17-OH-CS. A consistent rise in the concentration of free 17-OH-CS was observed following operative trauma. Were this apparent delay in the rate of free steroid metabolism the direct reflection of a delay in the rate of conjugation, and unassociated with a change in renal function, a decrease in the plasma concentration of conjugates would be anticipated. Such a decrease was not found. The results of this study, then, do not support the thesis that the concentration of conjugated 17-OH-CS in plasma reflects a change in the rate of free 17-OH-CS metabolism following surgical operation. Neither do they support the suggestion that the concentration of total 17-OH-CS (free plus conjugated) in plasma provides an index of adrenocortical secretion which is independent of change in the rate of intermediary metabolism of these hormones.

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# BINDING OF CORTICOSTEROIDS BY PLASMA PROTEINS. VI. THE BINDING OF CORTISOL AND ALDOSTERONE BY CORTICOSTEROID-BINDING GLOBULIN AND BY THE ESTROGEN-INDUCED BINDING SYSTEM OF PLASMA*

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ABSTRACT

The binding of cortisol-4-$C^{14}$ and aldosterone-$H^3$ by serum from pregnant or estrogen-treated subjects was directly compared to the binding by serum from control subjects in a system of double equilibrium dialysis. Increased binding of cortisol-4-$C^{14}$ could be demonstrated by "estrogen" plasma under conditions of minimal cortisol loading only at 37°C. and not at 4°C. This difference of binding behavior is evidence that the binding sites induced by estrogen are qualitatively different from those on normal corticosteroid-binding globulin. The total concentration of cortisol-binding protein induced by estrogens may be two or three times greater than that of corticosteroid-binding globulin. Under physiologic conditions of temperature, aldosterone-$H^3$ is bound primarily by albumin, but at 4° C. aldosterone-$H^3$ is bound about equally by albumin and corticosteroid-binding globulin. At 4° C. the estrogen-induced binding system does not have appreciable affinity for aldosterone-$H^3$.

THE active corticosteroid hormones, cortisol and corticosterone, circulate in the plasma largely as a complex formed with an $\alpha$-globulin which we have called the corticosteroid-binding globulin (CBG) (2, 3) and which Sandberg and Slaunwhite have called transcortin (4). When the plasma cortisol concentration is approximately 20 to 30 $\mu$g. per 100 ml., the binding sites of this protein are virtually saturated; only at higher concentrations is significant binding by albumin and other plasma proteins observed (2, 4–7). The extent of cortisol binding by plasma at physiologic levels of hormone is markedly influenced by temperature; less than 2 per cent of the plasma cortisone is unbound at 4° C., but at 37° C. from 5 to 10 per cent is unbound (4, 7).

Received June 9, 1960.

* Presented in part at the Conference on the Biological Activity of Steroids in Relation to Cancer, sponsored by Endocrinology Section, Cancer Chemotherapy, National Service Center, National Institutes of Health, Vergennes, Vermont, September, 1959 (1).

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Recently there have been reports on increased cortisol binding by plasma from pregnant women and from patients of both sexes receiving large doses of estrogens (7–11). The enhanced corticosteroid binding induced by estrogens has been attributed to a simple increase in the binding protein, but certain observations are at variance with this interpretation. When the amount of cortisol added to a dialysis equilibrium system at 4° C. was less than 0.5 μg. per 100 ml. of plasma, we were unable to detect increased binding of the hormone in the plasma of pregnant women (12). Because it is difficult to demonstrate more than the normal amount of binding in the customary equilibrium dialysis, a system of double equilibrium dialysis was developed which permits the direct comparison of the binding characteristics of two plasmas in exactly the same environment of diffusible steroids. When the binding of cortisol by the control plasma was directly compared to that by plasma from pregnant or estrogen-treated patients in this system, again there was little or no increased binding at 4° C. under conditions of minimal loading (13).

The binding of aldosterone by plasma proteins had received little attention. The extremely low level of circulating aldosterone and its short plasma half-life (14) suggest that it is not as extensively bound as is cortisol. Aldosterone has little affinity for the corticosteroid-binding site of CBG, as judged by the limited ability of *d,l*-aldosterone to displace corticosterone-4-$C^{14}$ and cortisol-4-$C^{14}$ from CBG in dialysis equilibrium (3).

In this paper, evidence is presented that the enhanced corticosteroid-binding activity of plasma in pregnancy or during administration of estrogen differs qualitatively from that of normal plasma. The pattern of binding of aldosterone has been compared to that of cortisol in both normal plasma and plasma from pregnant or estrogen-treated patients.

## MATERIALS AND METHODS

*Source of plasma.* The "control" plasma specimens were obtained from healthy adult male laboratory workers. The "pregnancy" plasma specimens were obtained from women in the third trimester of clinically normal, uncomplicated pregnancies. "Estrogen" plasma specimens were obtained from patients receiving large doses of estrogen and were provided for this study by Dr. Ann Carter of New York and the late Dr. Delbert Bergenstal of the National Institutes of Health. All the latter patients were suffering from carcinoma of either the breast or the prostate and were receiving more than 10 mg. of diethylstilbestrol per day, or a biologically equivalent dosage of other estrogens. The "estrogen" plasma used in the study of aldosterone binding was obtained from a woman with metastatic carcinoma of the breast before and after the daily administration of 5 mg. per day of diethylstilbestrol.

When blood was drawn in the foregoing cases, heparin was used as an anticoagulant. The plasma was stored at −10° C. until analyzed.

Lyophilized human serum albumin (Fraction V, Squibb) was dissolved in Krebs phosphosaline buffer, pH 7.4, to make a 4.5 per cent solution.

*Source of labeled steroids.* Cortisol-4-$C^{14}$ with a specific activity of 1.47 millicuries per millimole was provided by the Endocrinology Study Section of the National Institutes of Health. The radiopurity of this material was confirmed by paper chromatography. Aldosterone-$H^3$ with a specific activity of 0.74 microcurie per microgram, prepared by the Wilzbach procedure, was extensively purified by Dr. J. O. Davis of the National Heart Institute, Bethesda, Maryland.

*Equilibrium dialysis.* The conditions of dialysis, extraction of steroids and measurement of radioactivity of cortisol-4-$C^{14}$ have been described in detail in earlier publications (3). In double equilibrium dialysis, two cellophane bags (Visking Nojax casing, 18/32) each containing 5 ml. of the plasma samples to be compared, were added to a large test tube containing 80 ml. of phosphate-saline buffer to which had been added the labeled steroid under study. Unless otherwise indicated, 0.5 μg. of cortisol-4-$C^{14}$ and 0.013 μg. of aldosterone-$H^3$ were added for 10 ml. of total plasma in the system. The tubes were continuously inverted on a rotating wheel for forty-eight hours at 4° C. When the experiments were conducted at 37° C., the tubes were shaken in a water bath. The plasma proteins were generally measured before and after dialysis, but the degree of dilution of the plasma during equilibration was disregarded.

In the experiments with aldosterone-$H^3$, the extracted steroids were dissolved in a toluene-DPO (diphenyloxyzol), and POPOP (1,4-bis-2-(5-phenyl-oxazolyl)-benzene) phosphor. The radioactivities of aliquots of the toluene solution were determined with a liquid scintillation counter.

*Ultrafiltration.* Ultrafiltration was carried out according to the method of Prasad and Flink (15). A 10-ml. sample of plasma containing 0.0332 μg. of aldosterone-$H^3$ was centrifuged at 2500 rpm in an International centrifuge PR-2 for three hours at either 4° C. or 37° C. Aldosterone-$H^3$ was extracted with 5 volumes of chloroform. After treating the chloroform extract with anhydrous sodium sulfate, the solvent was removed under reduced pressure. The residues were dissolved in the phosphor and counted as described under "Equilibrium dialysis."

## RESULTS

### *Binding of cortisol by "pregnancy" and "estrogen" plasmas*

The binding of cortisol-4-$C^{14}$ by "pregnancy" and by "estrogen" plasma was compared to the binding by control plasma in the double equilibrium dialysis system. Confirming our earlier report, there was little difference in the binding of cortisol when experiments were conducted with 0.5 μg. of cortisol added per 10 ml. of total plasma at 4° C. (Fig. 1). When the same experiment was repeated at 37° C., a striking shift occurred in the relative binding of cortisol-4-$C^{14}$ by the two plasmas. At physiologic temperatures there was a great relative increase in the binding by the "pregnancy" and "estrogen" plasmas. The absolute binding decreased in both plasmas at 37° C. but the decrease was greater with control plasma.

A similar shift in the relative binding by "pregnancy" and "estrogen" plasmas occurred when 5.5 μg. of nonradioactive cortisol was added to plasma in double equilibrium dialysis experiments conducted at 4° C. (Fig. 1).

The relative binding by "estrogen" plasma was examined at 4° C. with

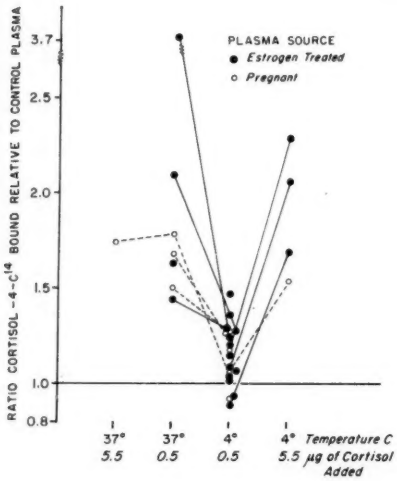


FIG. 1. Double equilibrium dialysis at 4° and 37° C. with two levels of cortisol loading. The binding of cortisol by "estrogen" or "pregnancy" plasma relative to the binding by control plasma is plotted on the ordinate.

a much larger range of cortisol additions. For this experiment, plasmas from 3 estrogen-treated patients were pooled. The concentration of 17-hydroxycorticosteroids in the pool was 65 µg. per tube, indicating the extent of the estrogen-induced changes in corticosteroid transport. The binding of cortisol-4-$C^{14}$ by this plasma pool was compared to binding by control plasma in 6 double equilibrium dialyses containing increasing additions of total cortisol from 0.25 µg. to 100 µg. per 100 ml. The results of this experiment are listed in Table 1. With the least amount of added cortisol

TABLE 1. EFFECT OF CORTISOL LOADING ON RELATIVE BINDING OF CORTISOL-4-$C^{14}$ BY POOLED "ESTROGEN" PLASMA AND CONTROL PLASMA

| Dialysis tube | Total µg. cortisol added | Unbound steroid (cpm/ml.) | Bound steroid (cpm/ml.) | | Relative binding activity* |
|---|---|---|---|---|---|
| | | | "Estrogen" plasma | Control plasma | |
| A | 0.25 | 1 | 61.4 | 47.3 | 1.29 |
| B | 0.5 | 1.4 | 101 | 76 | 1.33 |
| C | 1 | 3 | 212 | 146 | 1.45 |
| D | 5 | 40 | 924 | 383 | 2.41 |
| E | 10 | 120 | 1425 | 546 | 2.61 |
| F | 100 | 207 | 466 | 310 | 1.50 |
| G | 0.5 | 1.7 | 90 | 88 (Control) | 1.02 |

*Conditions:* Double dialysis equilibrium, 5 ml. of each plasma. The difference in counting rates (cpm/ml.) between tubes was determined by the amounts of cortisol-4-$C^{14}$ and cortisol added. In tube G, two samples of control plasma were added.

* Relative binding activity in this and subsequent tables is the quotient of the bound hormone in the test plasma divided by the bound hormone in the control plasma.

the "estrogen" plasma bound only 29 per cent more cortisol than did the control plasma. The relative binding by the "estrogen" plasma increased greatly as more cortisol was added to the system up to 10 μg. When 100 μg. of cortisol was added, a striking decrease in the relative binding of cortisol by the "estrogen" plasma occurred.

*Binding of aldosterone-H³ by human albumin and normal human plasma*

The binding of aldosterone-H³ by human plasma albumin was measured in single equilibrium dialysis using 5 ml. of 4.5 per cent albumin, 0.0065 μg. of aldosterone-H³ and 40 ml. of phosphosaline buffer. Both at 4° C. and 37° C., about 67 per cent of the labeled hormone was protein-bound. When this result is compared with those reported in the literature (16)—for cortisol (72 per cent), for corticosterone (85 to 94 per cent), and for cortisone (75 to 78 per cent)—it is evident that all three corticosteroid hormones are bound to albumin to a greater extent than is aldosterone.

Aldosterone-H³ was bound to a greater extent (Table 2) by normal plasma at 4° C. than by albumin, in both dialysis equilibrium and ultrafiltration experiments. This finding suggested that CBG also might bind aldosterone-H³ under these conditions. When the binding sites of CBG were saturated by the addition of 10 μg. of nonlabeled cortisol, there was a fall in the extent of binding from 88 to 62 per cent. When the same experiments were repeated at 37° C., cortisol loading decreased the extent of binding only from 63 per cent to 58 per cent.

The predominance of albumin binding of aldosterone at physiologic temperatures was checked with double dialysis equilibrium (Fig. 2). At 4° C., intact plasma (with an albumin content of 4.5 per cent) bound 2.8 times as much aldosterone-H³ as was bound by 4.5 per cent albumin. In

TABLE 2. BINDING OF ALDOSTERONE-H³ BY NORMAL PLASMA; EFFECT OF TEMPERATURE AND CORTISOL LOADING

| Method of measurement | | Aldosterone-H³ bound to protein (%) | |
|---|---|---|---|
| | | at 4° C. | at 37° C. |
| A. Equilibrium dialysis | 1 | 85.4 | 60 |
| | 2 | 88.4 | 64 |
| | 3 | 89.3 | 65.4 |
| | *Mean* | *87.6* | *63.1* |
| B. Equilibrium dialysis with 10 μg. of cortisol | 1 | 63.2 | 59 |
| | 2 | 60.0 | 58 |
| | 3 | 62.8 | 57.5 |
| | *Mean* | *61.6* | *58.2* |
| C. Ultrafiltration | | 86.7 | 63 |

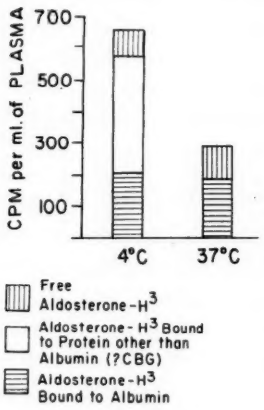


FIG. 2. Double equilibrium dialysis of normal human plasma and 4.5 per cent albumin at 4° and 37° C. The total height of each column is the total number of counts in the plasma sample. The upper lined portion of each column is the concentration of unbound aldosterone as determined in the dialysis buffer. The aldosterone bound to albumin was calculated as the concentration of bound aldosterone-$H^3$ in the 4.5 per cent albumin bag. The bound aldosterone-$H^3$ not associated with albumin at 4° C. is attributed to CBG.

TABLE 3. DOUBLE EQUILIBRIUM DIALYSIS STUDIES OF THE EFFECT OF PREGNANCY AND ESTROGEN THERAPY ON THE PLASMA PROTEIN-BINDING OF ALDOSTERONE-$H^3$ AND CORTISOL-4-$C^{14}$

| Donor of test plasma** | Temperature (C.) | Bound steroid (cpm/ml.)* | | Unbound steroid (cpm/ml.) | Relative binding |
|---|---|---|---|---|---|
| | | Test plasma | Control plasma | | |
| A. *Aldosterone-$H^3$ binding* | | | | | |
| E.H., pregnant | 4° | 184 | 228 | 54 | 0.81 |
| S.N., pregnant | 4° | 246 | 249 | 54 | 0.99 |
| I.P., pregnant | 4° | 307 | 384 | 51 | 0.80 |
| L.B., estrogen Rx | 4° | 463 | 505 | 70 | 0.91 |
| | | | | | *Mean 0.88* |
| E.H., pregnant | 37° | 97 | 129 | 80 | 0.75 |
| S.N., pregnant | 37° | 130 | 125 | 56 | 1.04 |
| I.P., pregnant | 37° | 126 | 140 | 70 | 0.90 |
| L.B., estrogen Rx | 37° | 325 | 305 | 125 | 1.06 |
| | | | | | *Mean 0.94* |
| B. *Cortisol-$C^{14}$ binding* | | | | | |
| E.H., pregnant | 4° | 277 | 234 | 13 | 1.18 |
| S.N., pregnant | 4° | 265 | 225 | 13 | 1.17 |
| | | | | | *Mean 1.18* |
| E.H., pregnant | 37° | 222 | 148 | 22 | 1.50 |
| S.N., pregnant | 37° | 201 | 120 | 21 | 1.68 |
| L.B., estrogen Rx | 37° | 231 | 142 | 7 | 1.63 |
| | | | | | *Mean 1.60* |

* The differences in counting rates between experiments are attributable to differences in counting conditions.

** Patients E.H. and S.N. were in the eighth month of pregnancy; Patient I.P. was in the ninth month of pregnancy; and Patient L.B. had received 5 mg. of stilbestrol for one month.

contrast, at 37° C. approximately equal amounts of aldosterone-$H^3$ were bound by the same plasma and by 4.5 per cent albumin.

*Binding of aldosterone-$H^3$ by "pregnancy" and "estrogen" plasmas*

Plasma obtained from women in the third trimester of pregnancy bound no more aldosterone-$H^3$ than did control plasma in double equilibrium dialysis at either 4° C. or 37° C. (Table 3). Similarly, at both temperatures, plasma from a patient receiving 5 mg. of diethylstilbestrol daily bound no more aldosterone-$H^3$ than did the control sample of her own plasma obtained before estrogen therapy. To confirm that an increase in cortisol-binding activity had actually occurred in the plasmas under study, the relative binding of cortisol-4-$C^{14}$ by 3 of the same plasmas was determined in the double equilibrium dialysis system. At 4° C. there was only a slight increase in relative binding by 1 of the 3 plasmas. However, at 37° C. all 3 plasmas bound about 60 per cent more cortisol than did the control plasma.

The effect of cortisol loading on the relative binding of aldosterone by "pregnancy" plasma was investigated because a significant increase in binding of cortisol-4-$C^{14}$ by pregnancy plasma is not demonstrable at 4° C. without loading. The results of double dialysis equilibrium with pooled pregnancy plasma and control plasma failed to show any change in the relative binding of aldosterone-$H^3$ by the two types of plasma after the addition of 10 μg. of nonradioactive cortisol (Table 4). For comparison, aliquots of the same plasmas were tested for relative binding of cortisol-4-$C^{14}$ under the same conditions. Cortisol loading increased the relative binding by the pooled "pregnancy" plasma to 75 per cent.

TABLE 4. DOUBLE EQUILIBRIUM DIALYSIS STUDIES OF ALDOSTERONE-$H^3$ AND CORTISOL-4-$C^{14}$ BINDING BY POOLED PREGNANCY PLASMA; EFFECT OF ADDITION OF 10 μG. OF NONRADIOACTIVE CORTISOL AT 4° C.

| Sample | | Unbound steroid (cpm/ml.) | Bound steroid (cpm/ml.) | | Relative binding |
|---|---|---|---|---|---|
| | | | Pregnancy plasma | Control plasma | |
| A. *Aldosterone-$H^3$ binding* | | | | | |
| Pooled pregnancy plasma | #1 | 70 | 150 | 146 | 1.03 |
| | #2 | 62 | 127 | 147 | 0.87 |
| | | | | | *Mean 0.95* |
| B. *Cortisol-$C^{14}$ binding* | | | | | |
| Pooled pregnancy plasma | #1 | 32 | 292 | 156 | 1.87 |
| | #2 | 31 | 306 | 187 | 1.64 |
| | | | | | *Mean 1.75* |

## DISCUSSION

These experiments clearly confirm the reported increase in cortisol binding at physiologic temperatures by plasma from pregnant and estrogen-treated subjects, and also explain the apparent contradictory results which we had obtained (13). The fact that the strength of CBG binding of cortisol is greatly increased at 4° C. whereas no such increase occurs in the estrogen-induced cortisol-binding system, permitted the measurement of CBG in the two types of plasma. The results indicate that there is little or no increase in CBG activity in pregnancy or after the administration of large doses of estrogens. When the CBG sites are occupied by cortisol following cortisol loading at 4° C., the predominance of secondary binding sites induced by estrogens is evident. From the cortisol-loading experiments, it is clear that the total amount of cortisol which can be bound by the estrogen-induced system is considerably larger than that which can be bound by CBG.

The plasma-protein binding of aldosterone is much weaker than that of cortisol. At 4° C. the affinity of CBG for aldosterone is sufficient to bind somewhat more aldosterone than is bound by albumin. The decrease in aldosterone binding by plasma at 37° C. as compared to 4° C. is attributable to a decrease in the binding of aldosterone by CBG with no change in the binding of albumin. Our findings would, therefore, indicate that albumin is the chief medium for transportation of aldosterone in the circulation.

The affinity of the estrogen-induced binding system for aldosterone is even less than that of CBG. When compared to control plasma, "pregnancy" and "estrogen" plasmas bound no more aldosterone under any of the conditions examined. The fact that cortisol loading at 4° C. failed to uncover increased aldosterone binding by "pregnancy" and "estrogen" plasmas relative to control plasma is in distinct contrast with the observed measurements of increased cortisol binding. This observation further supports the concept that the binding sites induced by estrogen differ from the sites on normal corticosteroid-binding globulin.

Attempts to separate estrogen-induced binding protein from normal corticosteroid-binding protein have thus far been unsuccessful. There is nothing in the present data which would be incompatible with the view that, as the production of binding protein is increased with estrogen, qualitative changes are induced in some binding sites that account for the altered behavior at 4° C.

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# THE QUANTITATIVE EXTRACTION AND PARTIAL PURIFICATION OF STEROIDS IN TISSUE AND BLOOD*

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ABSTRACT

A method for the quantitative extraction and partial purification of steroids in tissue and blood is described. The technique consists of extraction with 4:1 methylal-methanol, solvent partition (in certain cases only), and purification by means of a simple silica-gel column. Completeness of extraction was studied by means of radioactive steroids added to, perfused through, or incubated with tissues. Silica-gel purification results in recovery of 95 per cent or better of the steroids, with recovery of only 5 to 7 per cent of the original weight of the crude lipid extract.

RECENT years have witnessed continuing refinement of micro and ultramicro methods for the separation and measurement of steroids. Moreover, increasing attention is being paid to steroidogenesis in human tissues obtained for biopsy or at operation. In these situations the amount of tissue made available is extremely limited. Thus techniques for quantitative extraction of sub-microgram quantities of steroids from blood and tissue have assumed increasing importance. The very multiplicity of extraction procedures in current use for both small-scale and relatively large-scale work (1–12) indicates to some extent that none of them has proved uniformly applicable or satisfactory. Solvent partitions based on sound theoretical considerations and shown to be excellent when applied to pure steroids, may prove unsatisfactory when applied to crude extracts with a high fat content; in addition those solvent systems which have a tendency to produce emulsions may cause disastrous losses in the case of microgram quantities of steroids. Charcoal or other adsorption techniques often cannot be applied, and are at all events laden with the dangers inherent in such highly surfactant materials. Dialysis or dialysis-extraction procedures (5, 8, 11–14) have been used with considerable success, but are somewhat cumbersome and time-consuming. It is noteworthy, too, that many of the

Received May 13, 1960.

* This work was supported by grant A-767, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service.

extraction methods currently in use have not been properly evaluated with respect to their quantitative aspects.

Even in serum, where steroids are associated rather loosely with proteins, extraction problems may be difficult; for example, acetone extraction of serum leaves from 14 to 67 per cent of estrogen in the protein precipitate (9, 3). Szego and Roberts (3, 15, 16) found it necessary to extract sequentially with acetone, acetone-ether, ether at pH 1 after glucuronidase hydrolysis, again after strong acid hydrolysis, and finally after NaOH digestion in order to remove estrogen quantitatively from serum. Sandberg *et al.* (9) confirmed these findings. Such careful studies do not support the bioassay data of Bischoff *et al.* (17), who claimed that estrogen was extracted quantitatively from blood by precipitation in 4 volumes of ethanol. Bush (7) also emphasized the relatively poor recoveries of steroids from whole blood by the use of common extraction procedures. Simultaneous dialysis and extraction (13) of whole blood with methanol and methylene chloride is said to yield clean extracts with recovery of 84 to 100 per cent of added steroids. Unfortunately, in these studies estrogens, the most firmly protein-bound of the steroids, were not investigated.

Extraction of steroids from tissue rather than blood presents still more formidable problems due to the large amounts of accompanying fat. Ethyl acetate (12) or 1:3 ether-ethanol have been used, apparently with good recovery in some instances. Diczfalusy and Lindkvist (4, 18) carried out detailed studies of estrogen extraction from placental tissue, and found that exhaustive extraction with ethanol and butanol was unsatisfactory. Re-extraction after alkali hydrolysis was found necessary (as also noted with adrenal tissue by Salhanick and Berliner (19)), and the procedure of Ogata and Hirano (20) was found to yield additional estrogen even after the hydrolytic procedures of Szego or Rakoff *et al.* (21) had been applied. Alcohol extraction alone removed only 60 to 75 per cent of the tissue estrogen. Sandberg *et al.* also noted that liver tissue, incubated with radioestrone and phosphate buffer, and extracted with acetone and then with ether, did not liberate all the remaining estrogens upon mild (0.1 *N*) alkali hydrolysis.

In our own laboratory, experiments were undertaken to study the recovery of $C^{14}$-labeled steroids added to ground, fresh beef muscle and fat. Various techniques of acetone or alcohol extraction, with or without low-temperature precipitation of fats, failed to demonstrate satisfactory recovery or reproducibility when microgram quantities of steroids were added to amounts of tissue ranging from 10 to 100 Gm. Studies of estrogens added to blood and extracted by the method of Delsal (22) indicated that the steroid was not completely adsorbed to the protein precipitate but that direct extraction with methanol-methylal was quantitative. This extrac-

tion procedure has been evaluated with respect to $C^{14}$-labeled estrogens added to, perfused through, or incubated with tissues and blood from several species. By these devices we hoped to approximate the extractability of endogenous steroids, since absolute recovery studies of endogenous steroids at their normal tissue concentrations are not feasible. A silica-gel column chromatogram was then developed to separate the steroids from the large quantity of lipid material extracted. This eliminated the multiple separatory-funnel countercurrent techniques commonly used for defatting tissue extracts, and provided an extract suitable for further study. The diminution of pigments was advantageous, as well as the reduction in accompanying fats, which introduce appreciable variability into the Rf values of steroids chromatographed on paper.

## PROCEDURE

### I. *Extraction*

*Biologic fluids (whole blood, serum) are precipitated by pouring into 4 volumes of* Delsal's reagent (4 parts redistilled methylal to 1 part redistilled methanol). Tissues are *homogenized briefly in 4 volumes of the reagent. The mixture is shaken mechanically* or agitated frequently by hand for one hour and allowed to stand at room temperature *for another hour or overnight. The precipitate is collected by centrifugation or preferably* by suction filtration, and re-extraction is performed twice with the same volume of *solvent used previously. The extracts are combined and evaporated almost to dryness* at 40°C. (under nitrogen in the case of sensitive materials). A closed or a continuous-feed *rotating flash evaporator* (Laboratory Glass and Instruments Corp., New York, N. Y.) is convenient for large volumes.

### II. *Solvent partition*

This is an optional procedure, used under special conditions, as for the removal of $C^{14}$-labeled acetate, mevalonic acid, or water-soluble contaminants. The residue from the extraction is reconstituted to a convenient volume with the methylal-methanol reagent (20 to 200 ml., dependent upon the quantity of lipids extracted). To this solution in a separatory funnel are added 2.5 volumes of redistilled peroxide-free diethyl ether, 1 volume of redistilled peroxide-free isopropyl ether, and 1 volume of 1 per cent aqueous acetic acid. The mixture is shaken throughly and the lower aqueous phase discarded. The organic phase is washed twice more with ½ volume of distilled water and evaporated to dryness.

### III. *Defatting*

Disposable chromatographic columns are prepared by drawing out the end of a 25-cm. length of glass tubing (10 mm. outside diameter) to a fine capillary. The column is half filled with hexane and a plug of cotton is tamped into the bottom of the column. A slurry of 2 Gm. of silica gel in hexane (Davison silica gel, No. 923, 100–200 mesh) is poured into the column and settled by frequent tapping with a glass rod. Washed sea sand (a few mm.) is placed on top of the silica gel. The capillary is broken off at a point which permits a flow rate of hexane at about 0.5 ml. per minute. Such columns are suitable for processing up to about 0.4 Gm. (dry weight) of lipid extract. For larger quantities, the chromatograms are run in 25×300 mm. columns with a rotating Teflon valve, using 20 Gm. of silica gel.

The dried lipid extract is dissolved in 1–1.5 ml. of hexane and applied to the column after the supernatant hexane has run off. Another 5 ml. of hexane is used to rinse the sample container and is run through the column. The hexane eluates, which contain about 50 to 60 per cent of the weight of lipid applied, are discarded. Next, 35 ml. of 5 per cent ether-benzene (v/v) is run through the column to remove cholesterol and certain pigments; this fraction contains 10 to 15 per cent of the lipid applied. The final solvent phase is 30 ml. of ethyl acetate. The first portion of this solvent is added to the column when about 3 cm. of ether-benzene remains above the top of the silica gel; further small portions are added until the transition from one solvent to the other is complete. The ethyl acetate fraction is evaporated to dryness under nitrogen. For large-scale preparations involving the 25×300 mm. columns, the foregoing phase volumes are multiplied by 10. In dealing with extracts of blood, or of adrenal, testis, ovary or liver tissue, about 6 per cent of the weight of the dried lipid extract and 94 per cent or better of the tissue steroids are found in the ethyl acetate fraction.

## EXPERIMENTAL

### I. *Proof of quantitative extraction*

In this phase of the investigation only the completeness of extraction was studied. C-$^{14}$-labeled estrogens were selected as model steroids in view of evidence that these steroids are bound most firmly to proteins. Ideally, quantitative extraction studies should establish the distribution of the desired material in the various fractions obtained. In the present instance, however, it was impossible to determine the radioactivity of the crude lipid extract without adding steps to eliminate substances which impair the precision of radioactivity measurement through quenching or self-absorption. Such additional manipulations would entail unpredictable losses of material, and as a consequence the numerical values obtained for the distribution of radioactivity would lack the desired precision. Therefore, the efficiency of extraction was judged by the amount of radioactivity remaining in the final tissue residue.

*Estrogen added to tissue.* A 200-Gm. sample of bull testis parenchyma was homogenized in a Waring blendor with physiologic saline and adjusted to a volume of 200 ml. This material was divided into 4 equal portions and 11.0 μg. of 16-C$^{14}$-estradiol-17β containing 33,000 cpm was added to each. No carrier estradiol was added to the first portion, whereas the others received 0.09, 1.0 and 10.0 mg. respectively, dissolved in a minimum quantity of ethanol. The mixtures were shaken mechanically at room temperature for one hour. An equal volume of methylal-methanol (4:1) was added, and the mixture allowed to stand overnight in the refrigerator. The suspended tissue was filtered off by suction and subjected to re-extraction twice with a volume of methylal-methanol which was four times that of the original aliquot of testicular homogenate. The tissue residue was then treated with 200 ml. of 2.5 per cent NaOH at 37° C. until it had disintegrated completely. Following this process, the solution was adjusted to

a pH of 6 with HCl, and extraction was carried out four times with an equal volume of ether. The ether extract was dried with sodium sulfate, evaporated under reduced pressure, and diluted to 100 ml. with ethanol. Aliquots were taken for counting by liquid scintillation in a toluene-POP-POPOP[1] system (23) after evaporation of the alcohol. Quenching corrections were applied to all samples. These were determined by the addition of 25,000 cpm of $C^{14}$-benzoic acid in toluene to each sample vial as an internal standard, and re-counting. All samples were counted to a statistical accuracy of 5 per cent or better.

In this experiment, less than 0.4 per cent of the added radioactivity remained in the residue of the tissue aliquot without estradiol carrier, and less than 0.2 per cent of the added radioactivity was present in the tissue residues of each of the other 3 aliquots to which carrier estradiol had been added.

*Estrogen perfused through tissue.* A healthy adult stallion was given 3 consecutive daily injections of 40,000 IU of chorionic gonadotropin (APL, Ayerst). One day after the last injection, several liters of defibrinated blood were obtained; two days after the last injection, sterile castration was performed under intravenous chloral hydrate–magnesium sulfate anesthesia. The testis was irrigated with physiologic saline containing heparin; it was then cannulated, and incorporated in a perfusion system designed by L. R. Axelrod. Perfusion was carried out for ten and a half hours with 1.3 liters of blood containing penicillin and streptomycin. Fifteen minutes after commencing perfusion, a solution of 50 per cent aqueous ethanol containing 0.593 mg. of 16-$C^{14}$-estradiol-17$\beta$ with an activity of 1.82 million counts was infused over a period of fifteen minutes. At the end of the perfusion, the testis (weighing 186 Gm.) was homogenized in a Waring blendor with 200 ml. of methylal-methanol reagent. The residue, after suction filtration, was subjected to extraction as before with a total of 12 volumes of methylal-methanol. The final tissue residue was treated with NaOH. Following hydrolysis, the ether extract of the entire tissue residue showed a total radioactivity of 60 cpm, which was only twice the background count and therefore was statistically indistinguishable from zero.

*Estrogen incubated with tissue.* In the first experiment, 15-Gm. portions of guinea-pig liver slices were suspended in 100 ml. of Krebs-Ringer-bicarbonate buffer with added glucose and incubated at 37° C. for four hours in an atmosphere of 95 per cent oxygen and 5 per cent carbon dioxide. At the beginning of the incubation, 250,000 cpm of 16-$C^{14}$ estrone, estradiol-17$\beta$ or estriol were added. At the end of four hours, the samples were cooled to room temperature, homogenized, and treated by extraction as previously

[1] POP (2,5-diphenyloxazole), 4 Gm./L.; POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene), 0.05 Gm./L. Both were obtained from Pilot Chemicals, Inc., 36 Pleasant St., Watertown, Mass.

described. The tissue residue retained 1.3 per cent of the added estrone radioactivity, 0.4 per cent of the estradiol radioactivity and no detectable amount of the estriol radioactivity. A second series of incubations was prepared, using the technique described by Szego (3). Since these experiments demonstrated metabolism as well as binding of added estrogen, it was thought that incorporation of radioactive estrogen into the endogenous pool would be very probable, and that the results of extraction studies might therefore reflect the extractability of endogenous estrogen. Incubation of minced liver (0.2 Gm. of tissue per ml.) with 50,000 cpm of estrone per sample was carried out in serum, blood and saline for a period of two hours at 37° C. with shaking in an $O_2$-$CO_2$ atmosphere. Extraction was carried out as previously described. The radioactivity of the ether extract of the tissue residues after hydrolysis was at background levels in all the incubations.

II. *Partition of pure steroids in the solvent system*

The distribution of steroids other than cholesterol and the estrogens in Delsal's system methylal-methanol-aqueous acetic acid-diethyl ether-isopropyl ether has not been previously investigated. Since this procedure was of value in removing large excesses of $C^{14}$-acetate present in tissue incubations or organ perfusions, it was important to determine the partition of steroids of different polarities. This was accomplished most easily by the use of $C^{14}$-labeled or $H^3$-labeled compounds. Appropriate quantities of sodium acetate, benzoic acid, and a variety of steroids were dissolved in small volumes of methylal-methanol, and the partition with acetic acid and the two ethers carried out in separatory funnels under rigidly controlled conditions. All the extracts were brought to 10-ml. volumes in ethanol, and aliquots were evaporated to dryness in scintillation vials, dissolved in toluene, and counted before and after the addition of internal standards. The results are shown in Table 1. It may be seen that acetate is satisfactorily removed by this partition procedure. Most of the steroids show distribution coefficients strongly favoring the organic (upper) phase, with acceptable over-all losses in the aqueous fractions of less than 5 per cent. In the cases of estriol, 11$\beta$-hydroxy-$\Delta^4$-androstenedione, and particularly the more highly oxygenated $C_{21}$ steroids, the losses into the aqueous phases are substantial. Thus this partition procedure cannot be used indiscriminately. In situations where steroids rather than acetate, for example, are used as substrates, this partition is unnecessary.

III. *The defatting chromatogram*

Extracts of equine and bovine blood, liver, adrenal, testis and ovary were prepared in bulk as described, and kept in methylal-methanol solution. In many instances, old extracts which had become intensely pig-

mented with time were used purposely. Aliquots containing 0.2 to 0.3 Gm. (dry weight) of each lipid were pipetted into vials, and a volume of ethanol solution containing 50,000 cpm of the test steroid (used as received, without repurification) was added to each. Vials containing only the steroid were also prepared with each run. The mixture was dried *in vacuo*, weighed, and applied to the 2-Gm. silica columns in the manner described. The eluates were collected, dried, weighed and redissolved in ethanol. Suitable aliquots were pipetted into scintillation vials, dried, dissolved in toluene-POP-POPOP

TABLE 1. PARTITION OF STEROIDS IN THE SOLVENT SYSTEM

| Compound | Amount used | | Percentage found | | | | Total recovery |
|---|---|---|---|---|---|---|---|
| | Weight (μg.) | Activity (μc.) | In organic phase | In aqueous phase | In 1st $H_2O$ wash | In 2nd $H_2O$ wash | |
| Sodium acetate-1-$C^{14}$ | 11.3 | 0.17 | 5.1 | 72.0 | 12.4 | 9.9 | 99.4 |
| Benzoic acid-1-$C^{14}$ | 0.05 | 0.002 | 89.0 | 8.9 | 2.1 | 0.0 | 100 |
| Estrone-16-$C^{14}$ | 5.1 | 0.014 | 97.1 | 1.1 | 1.1 | 0.7 | 100 |
| 17β-Estradiol-16-$C^{14}$ | 11.1 | 0.03 | 98.5 | 0.8 | 0.4 | 0.2 | 99.9 |
| Estriol-16-$C^{14}$ | 2.1 | 0.048 | 89.9 | 4.6 | 2.1 | 3.4 | 100 |
| Testosterone-4-$C^{14}$ | 1.1 | 0.020 | 96.2 | 1.9 | 1.2 | 0.7 | 100 |
| $\Delta^4$-Androstenedione-3,17 | 1.2 | 0.0096 | 95.5 | 1.6 | 1.7 | 1.2 | 100 |
| 11β-Hydroxy-$\Delta^4$-androstenedione-3,17 | 17.3 | 0.085 | 84.7 | 5.8 | 5.4 | 4.0 | 99.9 |
| Progesterone-4-$C^{14}$ | 0.6 | 0.02 | 99.0 | 0.8 | 0.3 | 0.0 | 100.1 |
| 17α-Hydroxyprogesterone-4-$C^{14}$ | 0.4 | 0.014 | 95.7 | 1.1 | 0.7 | 2.5 | 100 |
| 11-Ketoprogesterone-4-$C^{14}$ | 17.8 | 0.025 | 96.3 | 1.3 | 1.6 | 0.8 | 100 |
| 3α,17α-Dihydroxypregnane-11,20-dione | 16.0 | 0.024 | 82.2 | 11.4 | 3.2 | 3.2 | 100 |
| Corticosterone-4-$C^{14}$ | 2.1 | 0.009 | 79.8 | 8.2 | 6.3 | 5.8 | 100.1 |
| Hydrocortisone-4-$C^{14}$ | 6.7 | 0.003 | 76.8 | 12.4 | 6.9 | 3.9 | 100 |
| Cholesterol-x-$H^3$ | 0.017 | 0.212 | 99.0 | 0.4 | 0.3 | 0.3 | 100 |
| Cholesteryl acetate-x-$H^3$ | 0.036 | 0.384 | 99.0 | 0.3 | 0.2 | 0.6 | 100.1 |

and counted to a statistical accuracy of 3 per cent. All samples were counted ten times to insure that replication was within acceptable limits. As an internal standard, 25,000 cpm of $C^{14}$-benzoic acid in toluene was then added, and the samples were re-counted. Arithmetic correction for quenching and self-absorption phenomena was made on the basis of the change in the known number of benzoic-acid counts added. In the hexane eluates from the columns the quenching and self-absorption were extremely high; in many instances the counts registered were less than five times background and thus within the range of statistical nonsignificance. The figures listed in Table 2 for the radioactivity in the hexane fraction are therefore likely to be too high. In fact they may be indistinguishable from zero, especially since other studies of scintillation counting in our laboratory have shown that simple arithmetic corrections become unreliable when the quenching and self-absorption is greater than about 10 per cent. Quenching in the other eluates was within acceptable limits. The data for small and large columns are shown in Table 2. The fractionation of the radioactivity was consistent throughout, regardless of the absence or presence of lipid, or its origin.

TABLE 2. PERCENTAGE RECOVERY OF RADIOACTIVE STEROIDS ADDED TO CRUDE LIPID EXTRACT

| Steroid added | Cholesterol-x-$H^3$ | Progesterone-4-$C^{14}$ | | Corticosterone-4-$C^{14}$ | Hydrocortisone-4-$C^{14}$ | $\Delta^4$-Androstenedione-4-$C^{14}$ | Estrone-16-$C^{14}$ | Estriol-16-$C^{14}$ | Estriol, testosterone, progesterone, hydrocortisone; all $C^{14}$ | Hydrocortisone-4-$C^{14}$ | Estradiol-17$\beta$-16-$C^{14}$ | Estriol-16-$C^{14}$ | Eluate residue. Per cent of applied weight in each eluate. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2-Gm. columns | | | | | | | | 20-Gm. columns | | | |
| Amount of tracer steroid ($\mu$g.) | 0.006 | 0.4 | 10.0 mg. | 6.0 | 0.5–0.9 | 1.0 | 9.0 | 2.0 | 1.0 | 0.4 | 0.02 | 1.6 | |
| Hexane | 0.40 | 0.13 | 0.13 | 2.11 | 2.5 | 0.44 | 0.03 | 0.05 | 1.4 | 0.95 | 0.1 | 0.5 | 50–60 |
| Ether-benzene | 102.3 | 0.12 | 0.43 | 0.0 | 0.48 | 0.0 | 0.04 | 0.04 | 0.0 | 2.3 | 2.9 | 0.0 | 10–15 |
| Ethyl acetate, first 10-ml. cut | 0.0 | 99.1 | 96.1 | 85.7 | 80.4 | 99.0 | 93.9 | 72.9 | 71.2 | | | 81.5 | |
| second 10-ml. cut | 0.0 | 0.14 | 0.18 | 7.3 | 10.8 | 0.06 | 0.0 | 26.8 | 20.2 | | | 15.2 | |
| third 10-ml. cut | 0.0 | 0.37 | 0.35 | 1.3 | 4.0 | 0.23 | 0.04 | 2.5 | 3.0 | | | 0.0 | |
| Total ethyl acetate eluate | 0.0 | 99.6 | 96.6 | 94.3 | 95.2 | 99.3 | 93.9 | 102.2 | 94.4 | 83.3 | 85.9 | 96.7 | 5–7 |

## DISCUSSION

The described procedure has been under study in our laboratory for the past several years and was tested for efficiency and practicality under the most severe conditions we could devise. Up to 2 liters of equine blood or several hundred grams of tissue were processed at one time with the aid of a continuous-feed flash evaporator. The avoidance of solvent partitions (except under special circumstances) and particularly of multiple-separatory-funnel or other countercurrent procedures has proved to be most advantageous. As many as 10 extracts can be defatted simultaneously when the small disposable columns are used. There is no exposure to extremes of pH and no materials more active than silica gel are used—a point which may be of importance in dealing with certain of the more sensitive steroids. The origin of the tissue extract did not appear to be of significance, for recoveries from the various extracts were identical even though the extracts differed considerably in their gross characteristics. There was also no significant difference in the corrected recoveries from duplicate lipid extracts of which half were first put through the solvent partition procedure. In a few instances, the ethyl acetate fraction from a hydrocortisone recovery experiment was re-run through another column, with 98 to 99 per cent recovery. This suggests that the original, commercial radiohydrocortisone may have contained some radioactive impurities resulting from self-irradiation breakdown.

*Acknowledgment*

We wish to express our appreciation to Dr. L. R. Axelrod for his participation in the testis perfusion study, and to Dr. M. A. Nyman for many helpful suggestions.

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# PREOPERATIVE TREATMENT OF THYROTOXICOSIS WITH ANTITHYROID DRUGS AND THYROXINE

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ABSTRACT

In thyrotoxicosis, it is probable that the thyroid gland is stimulated by some agent which operates without regard to the level of circulating thyroid hormone and that the level of normal TSH is depressed. When antithyroid drugs are administered the amount of circulating thyroid hormone decreases and the level of normal TSH increases, thereby causing enlargement of the gland. This investigation shows that administration of thyroxine prevents this increase in the size of the thyroid by keeping the patients euthyroid during continued treatment with antithyroid drugs. The variations in the size of the gland with different forms of treatment are discussed.

## INTRODUCTION

ANTITHYROID drugs are now used in many clinics for preoperative treatment of thyrotoxicosis. When these drugs are used alone, the thyroid gland becomes more vascular and friable, thereby increasing the technical difficulties during surgery and causing a higher incidence of complications. To avoid this, iodine is added to the therapy during the last two or three preoperative weeks. With the use of antithyroid drugs, the risks of surgery in less specialized clinics have been decreased significantly when compared to the risks with use of iodine alone (1).

After administration of antithyroid drugs the thyroid gland may enlarge, especially if the patient becomes hypothyroid, because of stimulation by the thyroid-stimulating hormone (TSH) of the anterior pituitary. In thyrotoxicosis, there is a high degree of thyroid activity despite a high level of circulating thyroxine. It is possible that the thyroid is being stimulated by some agent which operates without regard to the level of circulating thyroid hormone (2–5). When the amount of circulating thyroxine is decreased by antithyroid drugs, the amount of normal TSH increases, thereby causing an enlargement of the thyroid. This enlargement of the thyroid should be preventable by the administration of thyroxine after the

Received May 31, 1960.

patient has become euthyroid. The patient's own thyroxine production is thereby almost completely inhibited, and the euthyroid state can be maintained by the continued administration of thyroxine.

In this investigation, patients suffering from thyrotoxicosis were treated with antithyroid drugs until they became euthyroid. Then thyroxine was administered for two weeks, after which surgery was performed. After the operation, the dosage of thyroxine was gradually reduced until the patients had returned completely to a normal state.

A short preliminary communication has been published (6).

## MATERIALS AND METHODS

### *Patients and treatment*

During the period 1946–1959, 883 patients with hyperthyroidism were operated on at the Surgical Clinic I, Sahlgrenska Sjukhuset; there was one fatality postoperatively (in 1952). From 1946 to 1954, the preoperative management of thyrotoxicosis mainly consisted of the administration of iodine for a period of two to three weeks. From 1954 to 1958, antithyroid drugs were administered alone for four to eight weeks, and in combination with iodine during the two or three weeks preceding surgery. Since 1958, thyroxine has been administered during the last two weeks instead of iodine. Exceptions were made in cases in which treatment had been started by others. Since 1952 almost all the patients were operated on by the senior author, Bergfelt. Only these cases are included in this report.

To obtain uniform material, only the results in the cases of toxic diffuse goiter are presented. These cases were divided into 5 groups: *Group A*, pretreated with iodine, 237 patients; *Group B*, pretreated with antithyroid drugs plus iodine, 121 patients; *Group C*, pretreated with antithyroid drugs plus thyroxine, 47 patients; *Group D*, pretreated with antithyroid drugs plus iodine plus thyroxine, 5 patients; and *Group E*, the control series, 57 patients suffering from nontoxic goiter in whom the surgical procedure was similar to that in the other groups.

The following drugs were used:

*Iodine solution:* iodine, 5 Gm.; potassium iodide, 10 Gm.; and distilled water, 85 ml.

*Thyroxine: l*-thyroxine-Na corresponding to 0.1 mg. of *l*-thyroxine (Levaxin®, Nyegaard et Co. A/S, Oslo, Norway).

*Antithyroid drug:* 1-methyl-2-mercaptoimidazol, 5 mg. (Thacapzol®, AB. Kabi, Stockholm, Sweden).

*Group A* received 10 drops of iodine solution 3 times a day for about two weeks. In some cases, the treatment was continued for another week with 20 drops of iodine solution three times a day.

*Group B* received 5–10 mg. of methyl-mercaptoimidazol four times a day until euthyroid, usually after four to eight weeks. At this time a dose of 10 drops of iodine solution was administered three times a day, and the two drugs were adminstered together for two or three weeks before operation.

*Group C* received methyl-mercaptoimidazol as in Group B. When a euthyroid condition was produced, 0.2 mg of thyroxine was administered daily during the last two weeks before the operation. In some cases the dose of methyl-mercaptoimidazol was reduced before thyroxine was added.

*Group D* received methyl-mercaptoimidazol and iodine solution as in group B. Together with the iodine solution, thyroxine was administered as in group C.

*Group E* received no preoperative treatment.

*Time in hospital*

Patients in Group A were in the hospital during the whole preoperative period, usually for two to three weeks. During the period 1954–1955, patients in Group B were hospitalized as in Group A; after that time they were admitted to the hospital as in Group C. Patients in Groups C, D and E were admitted to the hospital the day before operation. All groups were hospitalized for about five to six days postoperatively.

*Technique of operation*

The operations were routinely performed as follows: The prethyroid muscles were divided, and exposure of the thyroid obtained by a wide lateral dissection. The superior thyroid vessels were ligated and divided, whereas the inferior thyroid arteries were ligated in continuity. Two or more parathyroids, as well as the recurrent laryngeal nerves, were exposed. The major portions of both lateral lobes and all of the isthmus and pyramidal lobes were resected. The 3–4 Gm. portions of each lateral lobe which were allowed to remain, were folded over to the laryngeal and tracheal fascia. The weights of the removed parts were fairly representative of the size of the glands. The wounds were routinely closed with drainage.

*Pathologic examination.*

Tissue slices were examined with a microscope for the amount of colloid in the glands and for the height of the cells in the follicles.

The pathologic examination did not include all of the material. Thirty-three patients from the year 1953, when the administration of iodine as preoperative treatment was common, were selected as representative of Group A; 34 patients from the period 1956–1957, when preoperative treatment with antithyroid drugs and iodine was common, were selected as representative of Group B; 40 cases from the period 1958–1959 represented Group C; and all 5 cases in Group D were examined.

## RESULTS

The average distribution of age, initial basal metabolic rate (BMR), and weight of the removed thyroid tissue are shown in Table 1. Morning pulse rates for each group of patients on the day of operation and on the following three days are shown in Figure 1.

TABLE 1. THE AVERAGE DISTRIBUTION OF AGE, INITIAL BASAL METABOLIC RATE AND WEIGHT OF THE REMOVED THYROID TISSUE

| Group | Age (yrs.) | Initial BMR (%) | Weight of removed thyroid tissue (Gm.) |
|---|---|---|---|
| A | 37 | 55 | 37 |
| B | 40 | 56 | 44 |
| C | 45 | 51 | 25 |
| D | 39 | 54 | 37 |
| E | 50 | — | — |

*Age and BMR*

The average age was higher in Group C than in the other groups, owing to the fact that during recent years, indication for operation has been somewhat altered. Young patients with a small thyroid gland and mild hyperthyroidism were previously operated on, but are now treated with antithyroid drugs without surgery. There were no real differences in BMR between the different groups.

*Weight of thyroid tissue*

The weight of the removed thyroid tissue was fairly representative of the size of the gland, because the amount that was allowed to remain after surgery was the same in each patient. The weight was significantly less in Group C than in the other groups. The highest weight was in Group B. Weights were similar in Groups A and D.

*Body temperature and pulse*

There was no real difference in temperature reaction between the groups. The highest temperature was noted the first day after operation. It might

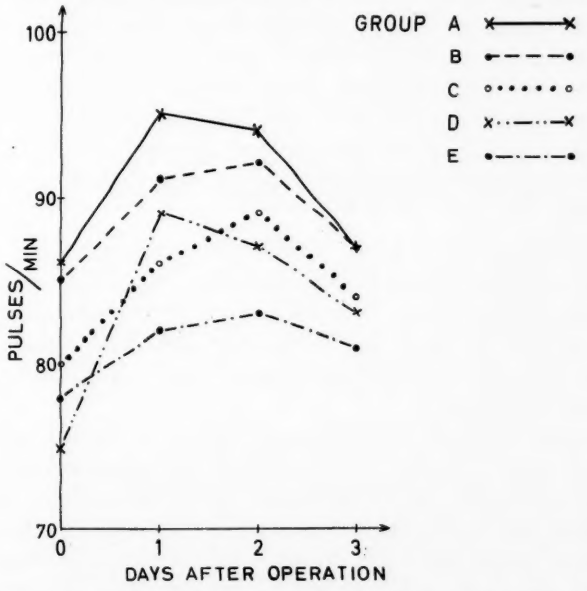


FIG. 1. Average morning pulse rates on the day of operation and on the following three days for Group A (iodine), Group B (methyl-mercaptoimidazol+iodine), Group C (methyl-mercaptoimidazole+thyroxine), Group D (methyl-mercaptoimidazol+iodine +thyroxine) and Group E (control).

be expected that the pulse would be higher in Group C than in the other groups because of the administration of thyroxine. However, the pulse rate in Group C was lower than in Group B and significantly less than in Group A.

*Pathologic examination*

The histologic picture varied within each group, but there were marked differences between the groups. The photomicrographs shown in Figure 2 are fairly representative for each group.

In Group A, no glands were devoid of colloid; the colloid content was low in 12 per cent, medium in 52 per cent, and high in 36 per cent. In Group B also, no glands were devoid of colloid; the content was low in 21 per cent, medium in 29 per cent and high in 50 per cent. In Group C the colloid content was nil in 34 per cent of the glands, low in 29 per cent, medium in 32 per cent, and high in 5 per cent. Thus, the amount of colloid was lowest in Group C.

In Group A the follicles were lined with cuboidal cells in 59 per cent of the glands, cuboidal to low columnar in 38 per cent, and low columnar to high columnar in 3 per cent. In Group B the follicles were lined with cuboidal cells in 28 per cent of the glands, cuboidal to low columnar in 9 per cent, and low columnar to high columnar in 63 per cent. In Group C the follicles were lined with cuboidal cells in 8 per cent of the glands; cuboidal to low columnar in 38 per cent, and low columnar to high columnar in 54 per cent. Thus, the cell-height was lowest in Group A.

Group D varied greatly in the foregoing respects, but was fairly similar to Group B.

## DISCUSSION

The effect of iodine has been investigated by many authors and has been summarized in earlier reviews (*e.g.*, 3). It is evident that iodine inhibits the release of thyroid hormone, at least for periods of two to three weeks, and thus causes an increase of colloid in the follicles. The BMR can be reduced by 20–30 per cent (7, 8).

Antithyroid drugs, such as thiouracil and mercaptoimidazol, inhibit the synthesis of thyroxine (9, 10). The release of thyroid hormone is not inhibited and thus the follicles are emptied. When iodine is administered there is again an increase of colloid in the follicles. The BMR can be decreased to a desirable level. By excessive treatment with antithyroid drugs, the patient will become hypothyroid, and the gland will increase in size because of stimulation by TSH.

When antithyroid drugs and thyroxine are administered, there is no inhibition of the release of hormone, and thus the follicles are emptied and

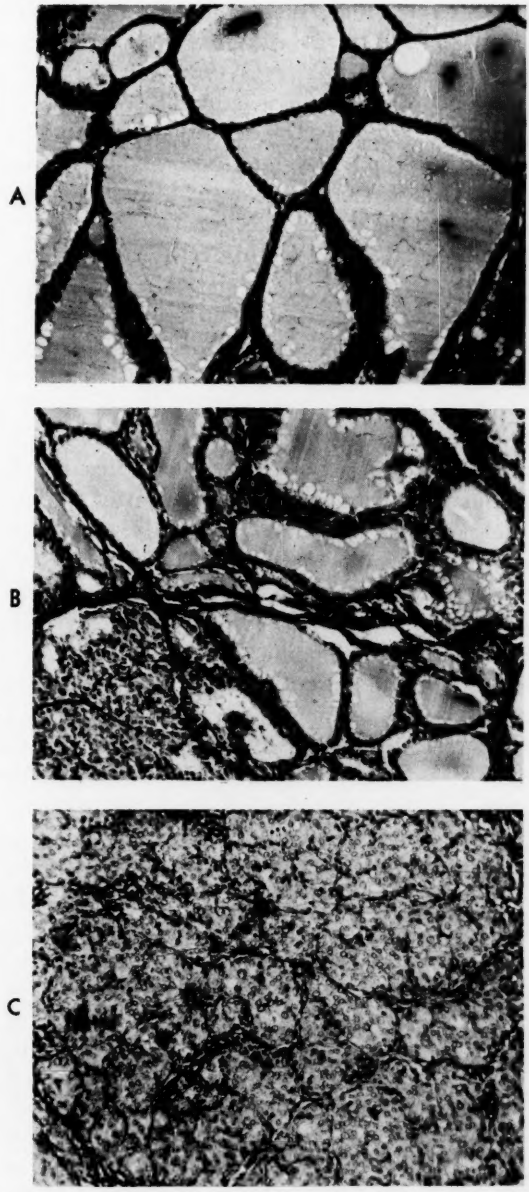


FIG. 2. Representative histologic pictures of the thyroid gland for Group A, Group B and Group C.

the gland decreases in size. The BMR is reduced by the administration of antithyroid drugs, and is kept at a desired level by the addition of thyroxine.

If enough exogenous thyroxine is administered, the cells lining the follicles ought to be cuboidal. In this investigation it is probable that the dosage of thyroxine was too low to inhibit normal TSH completely. There was no real difference between the heights of the follicle cells in Groups B and C. Group A showed the lowest cell-height.

However, the clinical picture was different. No patients in Group C showed signs of hypothyroidism. Some patients remarked that, after thyroxine was administered, the feeling of pressure from the thyroid disappeared. During the administration of antithyroid drugs, exophthalmos increased in a few patients; in some, there was an increased flow of tears; and 1 patient suffered from paralysis of the eye muscles. These symptoms disappeared when thyroxine was administered.

It is noteworthy that no patient experienced any untoward symptoms from the thyroxine medication, either before or after operation.

Patients with a large thyroid gland, especially when intrathoracic, were previously treated with iodine instead of antithyroid drugs. This study shows that it might be better to treat these patients with antithyroid drugs plus thyroxine, because the gland becomes smaller with this form of preoperative treatment than with iodine.

Group D included only 5 patients. However, this group showed a higher incidence of postoperative reactions than did Group C.

In Group E, the control, postoperative reactions were significantly less than in the other groups. This indicates the possibility of achieving a still lower incidence of postoperative reactions in thyrotoxicosis.

In the preoperative management of thyrotoxicosis, if the function of the thyroid gland is further reduced by more intensive treatment with antithyroid drugs plus thyroxine, postoperative reactions will probably occur less frequently.

*Acknowledgment*

The authors wish to express their deep gratitude to Professor Einar Ljunggren, Head of the Department of Surgery 1, Sahlgrenska Sjukhuset, Göteborg, for his interest and continued support throughout the work. The support of Professor Hugo Theorell, Head of the Biochemical Department, Nobel Medical Institute, Stockholm, is gratefully acknowledged. We also thank Dr. Bengt Artén for valuable assistance.

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# INTERMITTENT PRECOCIOUS BREAST DEVELOPMENT ASSOCIATED WITH MILD BRONCHITIS*

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ABSTRACT

The case is presented of a 4-year-old girl who experienced periodic episodes of mammary enlargement and slight genital precocity with hypersecretion of gonadotropin and estrogen, during attacks of mild respiratory infection. When in good health the patient did not present any evidence of unusual elaboration of estrogen, and the earlier manifestations of precocious sexual development subsided. A review of the association of pulmonary diseases with gynecomastia is presented. The possible causes of such mammary development are discussed, based upon the premise that the stimulus for the development originates in the lungs. The hormonal changes following this stimulus and the sentivity of the breasts to these factors are also considered.

BREAST development normally takes place in the prepubertal girl between the ages of 10 and 12 (1) and usually heralds other secondary sexual development, eventually leading to the menarche.

When budding of the breasts occurs at a significantly earlier age than normal, it is frequently an indication of impending sexual precocity, which may then proceed in an orderly manner as precocious development of all the secondary sex features, followed in turn by a premature menarche. At times, however, the premature breast development may be an isolated finding, unassociated with any evidence of genital maturation. When this occurs spontaneously, it is termed "premature thelarche" (1). The precipitating factors in such premature thelarche are unknown.

In males, breast development has been termed "gynecomastia." Although transient gynecomastia may occur in about one-third of pubertal boys, it may also occur secondarily to systemic disease or to ingestion of sex steroids at any age. Thus, when presented with the problem of breast tissue appearing in any patient, male or female, at a chronologic age at which one might not normally anticipate this development, the physician

Received May 26, 1960.

* This work was supported in part by a grant from the U. S. Public Health Service—Grant RG-5162(C1).

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must consider one of the following potential diagnoses: 1) sexual precocity, 2) premature thelarche, 3) hormone-secreting tumor or abnormal gonadal function, 4) breast development secondary to ingestion of a sex steroid, or 5) breast development secondary to certain systemic diseases. In Table 1 is an outline of the causes of abnormal mammary development.

The systemic diseases reported to be associated with gynecomastia or precocious breast development are those involving the liver, the lungs, and the central nervous system. The role of hepatic disease in initiating abnormal breast development is thought to be that of inability of the diseased liver to metabolize and conjugate sex steroids for urinary excretion. The resultant increase in the circulating level of unbound estrogens is believed to account for the gynecomastia in males or for the precocious breast development in females (2). Certain diseases of the central nervous system may induce sexual precocity through stimulation of hypothalamic-hypophyseal pathways, causing premature release of gonadotropins. This has been seen in cases of cerebral neoplasm, granuloma, or even after some of the viral encephalitides. It has also been postulated that the premature release of gonadotropins in children is effected through suppression or ablation of a gonadotropin-inhibiting factor—perhaps from the pineal gland—which normally inhibits the release of gonadotropin from the pituitary before puberty (1). This latter theory has been used to explain the coincidence of sexual precocity with pineal tumors. Wilkins, however, has suggested that even in pineal tumors, the precocity is not a result of ablation of functioning pineal tissue by the tumor, but rather the result of pressure stimulation of adjacent hypothalamic tracts by the enlarging neoplasm (1). In any event, although the exact initiating mechanism of sexual precocity due to intracranial factors is not known, once it does occur, sexual development follows a normal, progressive pubertal pattern which is only unusual by virtue of the early chronologic age of the patient at the time it begins.

The third organ system indicted as a source of stimulation leading to abnormal breast development, is the respiratory system. Breast development, particularly gynecomastia, has been described in association with pulmonary diseases of various types. The mammary hyperplasia and hypertrophy are frequently an isolated finding, without evidence of systemic estrogenic effects. Moreover, most of the reported cases of breast enlargement secondary to pulmonary disease have been in adult males, few of whom have shown systemic manifestations of hyperestrogenism.

This presentation deals with a case of precocious breast development apparently related to pulmonary disease. A review of the literature on this phenomenon is also presented. The case to be described is of unusual interest because development of the breasts occurred in an immature female

TABLE 1. CONDITIONS ASSOCIATED WITH ABNORMAL MAMMARY DEVELOPMENT

| Drug therapy | Tumors | Testicular alterations | Pituitary syndromes | Other endocrine diseases | Neurologic syndromes | Hepatic and alimentary alterations | Pulmonary alterations |
|---|---|---|---|---|---|---|---|
| Estrogens<br>Androgens<br>Desoxycorticosterone<br>Corticoids<br>Chorionic gonadotropin<br>Digitalis<br>Isoniazid<br>Amphetamine | Chorionepithelioma<br>Hydatid mole<br>Teratoma<br>Testicular interstitial-cell tumor<br>Testicular Sertoli-cell tumor<br>Adrenal tumor<br>Renal tumor<br>Mediastinal dysembryoma | Eunuchoidism<br>Castration<br>Klinefelter's syndrome<br>Hermaphrodism<br>Pseudohermaphrodism<br>Chronic infection (tuberculosis, lues, leprosy)<br>Trauma | Acromegaly<br>Gigantism<br>Cushing's syndrome<br>Chromophobe tumors | Adrenal hyperplasia<br>Hyperthyroidism<br>Diabetes mellitus | Parkinsonism<br>Syringomyelia<br>Meningoencephalitis<br>Skull trauma<br>Transverse myelitis<br>Medullary trauma<br>Paraplegia | Cirrhosis<br>Infectious hepatitis<br>Chronic hepatitis<br>Starvation<br>Dietary deficiency | Tumors<br>Tuberculosis<br>Bronchiectasis<br>Hydatid cysts<br>Abscesses<br>Polycystic lungs<br>Spontaneous pneumothrax<br>Nonspecific lung infection |

coincident with attacks of bronchitis, and the precocious mammary growth promptly subsided with resolution of each respiratory infection. In addition, systemic hormonal changes were studied both during the phase of activity and quiescence.

## CASE REPORT

*V.V.*, a 4-year-old white girl, was first referred to the endocrine clinic with the chief complaint of breast tenderness and enlargement. The patient's history up to this point was uneventful, in that she was born of a full-term pregnancy by normal delivery and was breast fed during the neonatal period. Growth and development during the first four years of life were perfectly normal and uneventful.

The family history revealed pulmonary tuberculosis in 1 brother. For this reason the patient's chest was examined by x-ray and she was tested with P.P.D. in November 1954, when she was 23 months old. The tuberculin test yielded negative results. The chest roentgenogram, however, had revealed increased peribronchial markings in both lungs, with no infiltration or consolidation in the parenchymal tissue; the cardiac silhouette was normal. A chest roentgenogram in December 1954 showed no change from the initial film.

### *First enlargement of breasts*

Except for some bronchitis during the winter of 1956, the patient had no specific complaints until February 1957 when, after a "chest cold," tenderness and enlargement of both breasts were noted. Three weeks after the onset of this complaint the patient was brought to the clinic.

*Physical examination* revealed a 4-year-old girl with normal somatic development for her age. She was 40½ inches high and weighed 42 pounds. All other findings were normal except for 1) the presence of glandular tissue in both breasts, without pain or tenderness at this time (three weeks after the initial enlargement); and 2) excessive labial development for her age, with vulvar lanugo hair but no clitoral enlargement.

*Laboratory studies.* A chest roentgenogram showed prominence of the bronchovascular markings bilaterally, unchanged from previous films. Bone age was in accord with the chronologic age of the patient. A skull roentgenogram showed a normal sella turcica. In a vaginal smear, the cells exhibited the cornification observed in normal *adult* females. Urinary 17-ketosteroid excretion was 1.6 mg. per twenty-four hours.

The child received no therapy, and when seen one month later the breasts had decreased so much in size that no glandular tissue was palpable. It is noteworthy that the vaginal smear at this time was of the castrate type to be expected in a 4-year-old girl.

### *Second enlargement of breasts*

In June 1957 the patient had another chest cold, followed after three or four days by breast enlargement and a whitish vaginal discharge. At this time she weighed 43½ pounds and her height was 42½ inches. Bone age was interpreted as one year more than her chronologic age. A chest roentgenogram showed no unusual changes compared to the previous films. Urinary gonadotropin assay, using the mouse uterine-weight technique, yielded a value greater than 26 but less than 52.8 mouse units per twenty-four hours (normal for the adult) and urinary 17-ketosteroids were unchanged. The vaginal smear showed normal estrogenic activity for an adult female.

No treatment was given, and one month later the breast enlargement had again completely disappeared, coincident with resolution of the respiratory infection. The vaginal smear at this time again showed the complete estrogenic deficiency for a child of this

age. Urinary estrogens were greater than 3 but less than 4 rat units per twenty-four hours (normal for children).

These findings were still normal when rechecked one month later and ten months later, during which period the patient had had no respiratory symptoms and no recurrence of breast enlargement.

*Third enlargement of breasts*

In December 1959, when she was 6 years and 11 months old, she had a cold and cough of the tracheo-bronchial type for three days. After this the mother again noted distinct breast enlargement with tenderness, and a whitish vaginal discharge. On physical examination, the external genitalia were more mature than normal for her age and the vaginal mucosa was very moist. The rest of the physical findings were normal. In December the vaginal smear showed only minimal estrogenic deficiency, which represented a marked increase in mature vaginal cells for her age.

She underwent another examination in February 1960, at which time the glandular breast tissue had disappeared. The vaginal mucosa was very dry and the cells were of the noncornified castrate type. The urinary gonadotropin titer was less than 6.6 mouse units per twenty-four hours, and urinary total estrogens were less than 5.6 rat units per twenty-four hours—normal values for the immature prepubertal child. Urinary 17-ketosteroids were 2.0 mg. per twenty-four hours. A roentgenogram of the wrist showed that the bone age was in accord with the chronologic age of the patient.

## DISCUSSION

Mammary development has been reported frequently in males in association with lung disease of varying pathogenesis. Del Castillo and associates (3) in 1941 presented 3 cases of gynecomastia and milk secretion associated with lung carcinoma in men 38, 44 and 47 years old, who also had hypertrophic pulmonary osteoarthropathy, decreased libido, feminine hair distribution, and a normal penis and testes. X-ray therapy for the lung carcinoma resulted in remission of the pulmonary symptomatology and total disappearance of the gynecomastia.

Three years later del Castillo *et al.* (4) presented another case of breast development in a 63-year-old man who had a broncho-alveolar epithelioma, osteoarthropathy, decreased libido, feminine hair distribution, and low urinary 17-ketosteroids. Three other similar cases were described by Alicia Sequera Tamayo in 1947 (5). In 1949 Maggi and co-workers (6) reported a male patient, 62 years old, with broncho-alveolar carcinoma and emphysema followed by gynecomastia.

Bariéty and Coury in 1950 (7), Huguenin *et al.* in 1954 (8), and Lachapele *et al.* (9) and Podestá (10) in 1958 presented other cases of adult males with lung tumors or abscesses associated with gynecomastia, and in many instances with pulmonary osteoarthropathy. When x-ray therapy was successful in producing resolution or remission of the pulmonary pathologic condition, there was also resolution or remission of the abnormal breast enlargement.

In 1957 Vanni and Secchi (11) reported a case of primary gastric carcinoma with liver and lung metastases in a man who also had gynecomastia.

A review of these reported data revealed 22 cases of pulmonary neoplasm with gynecomastia in which the appearance of the breast enlargement was correlated with the appearance or progression of the tumor. Moreover, in the first 3 patients of del Castillo *et al.* and in the 2 patients of Huguenin *et al.*, the breast development regressed in association with diminution of the primary lung tumor following x-ray therapy.

Gynecomastia following tuberculous infection has also been reported. Wilkins (1), on the basis of Dr. Edith Lincoln's study, described gynecomastia in children who had miliary tuberculosis successfully treated with streptomycin and thiazosulfone. All of these children showed evidence of precocious sexual puberty. In 1952 Crespo Santillana (12) presented the case of a 9-year-old boy with gynecomastia following miliary tuberculosis of the lung, who also had cryptorchism and normal 17-ketosteroid excretion.

In 1938 Altschul (13) described a case of diffuse pulmonary tuberculosis, bronchiectasis, lung carcinoma and gynecomastia. However, in this instance it is difficult to establish whether or not the gynecomastia was related to the non-neoplastic or to the neoplastic lung disease, or to both.

Other authors (Guinet *et al.* (14) and Molina *et al.* (15)) have reported additional cases of gynecomastia in tuberculous patients, but only after treatment with isoniazid (INH). In all of these patients the urinary 17-ketosteroids were low. Interruption of the INH therapy in one patient (Guinet *et al.* (14)) resulted in disappearance of the gynecomastia with progression of the acid-fast infection.

Gynecomastia has also been noted, albeit infrequently, in some rare pulmonary diseases. In 1932 Secco (16) described 4 patients who had gynecomastia appearing in association with pulmonary abscess, suppurated hydatid cyst, spontaneous pneumothorax, and a gun wound in the left lung, respectively. In 1951 Boulard *et al.* (17) reported a 20-year-old man with gynecomastia, who had polycystic lung disease, periostitis, female distribution of hair, relatively small testes, and low urinary 17-ketosteroids. In the same year Schermann (18) presented a case of bronchiectasis, pulmonary emphysema and right gynecomastia in a 62-year-old man in whom spontaneous regression of the breast tissue occurred without alteration in the pulmonary disease. In 1954 Wheeler *et al.* (19) reviewed a series of 160 patients with gynecomastia; there were 4 cases of bronchogenic carcinoma, 1 of lung abscess, 2 of bronchiectasis, 1 of empyema, 2 of pulmonary tuberculosis, 1 of pneumonitis and atelectasis associated with anterior poliomyelitis, and 2 of equivocal tuberculosis and bronchiectasis. All but 5 of the patients had normal urinary 17-ketosteroids; in these 5 patients (1

with empyema and 4 with lung carcinoma) 17-ketosteroid excretion was low.

In 1957 Bongiovanni and Eberlein (20) reported the case of a Negro female child, 5 days old, admitted to the hospital for pulmonary infection; she had evident breast enlargement, which disappeared five months later.

Our case is similar, in that precocious breast development occurred in a female child, 4 years old, following a very mild respiratory infection. However, our case differs, in that the hormonal changes were studied during the temporary period of breast enlargement. All signs of breast development disappeared when the pulmonary manifestations of the disease subsided.

The underlying mechanisms involved in the patient's periodic breast development associated with slight development of the external genitalia, is not clear. From the history it is evident that the appearance of breast tissue and associated cornification of the vaginal mucosal cells correlated well with the appearance of the chest colds. Mammary development started a few days after the beginning of each respiratory illness and spontaneously disappeared during the periods of good health. Although minimal, it represented true glandular development associated at times with definite tenderness. The enlargement of the external genitalia was slight, but was also evident only during the period of illness.

On one occasion the breast development could be correlated with increased urinary excretion of gonadotropin, as well as with increased secretion of estrogen as revealed by examination of the vaginal smear. During periods of infection the vaginal cells were usually typical of those in the adult female; but during the periods of good health, they regressed to the estrogenic-deficient phase characteristic of the immature female.

The urinary 17-ketosteroids were always in the range of normal for the chronologic age. Bone age was virtually normal, except for the one occasion on which slight evidence of acceleration was noted. A skull roentgenogram was interpreted as normal. These findings offered no clue as to the etiology of the precocious sexual puberty.

From the data it would seem that the patient experienced premature secretion of gonadotropic hormone, which was released under the stimulus of an unknown pulmonary factor, to induce transient ovarian stimulation. Apparently neither the pulmonary infection nor the release of gonadotropin was sufficiently prolonged to maintain the sexual precocity. The periodic excess of estrogen was not adequate to produce endometrial proliferation or detectable menstrual flow. However, it was sufficient to cause vaginal cornification and to stimulate enough breast growth to be detected clinically. It is possible, that the mammary tissue was particularly sensitive to

low levels of estrogens. The phenomenon of tissue sensitivity can no doubt explain some cases of unilateral gynecomastia or breast hypertrophy.

It is impossible to identify the pulmonary factor which stimulates either the diencephalon, or the pituitary directly, to bring about the precocious release of gonadotropins. There have been many attempts to explain this bizarre relationship of various types of pulmonary disease to mammary development.

Del Castillo (3) suggested that the tumor may 1) spontaneously produce an hormonal secretion which stimulates the mammary glands directly, or 2) produce an unknown factor which affects the breast via the pituitary, since the pituitary is considered essential for breast development. Although Villiaumey (21) and Turner (22) reported that the pituitary, under the influence of estrogen and progesterone, secretes a substance or substances which are essential for the development of the breasts, Nelson (23) demonstrated a mammary response to estrone and progesterone in the absence of a hypophysis in animals. These pituitary factors (mammotropin A released with estrogens and mammotropin B released with progesterone) could work either synergistically with the ovarian hormones or in themselves be the agents for stimulating the breast tissue (22). In 1944, del Castillo *et al.* (4) reaffirmed their theory of neoplastic hormonal secretion, based upon high levels of estrogens found in 3 patients with pulmonary neoplasm. This concept was supported by Maggi *et al.* (6), who suggested that the gynecomastia was due to an abnormal estrogen/androgen ratio.

Alicia Sequera Tamayo (5) also supported the theory of estrogen secretion by neoplastic growths. However she also suggested that an unknown factor originating from the lung carcinoma may be the means of stimulating the secretion of estrogens from the adrenal cortex, ovaries or testes.

Vanni and Secchi (11) had two different theories: the first was the possibility of congenital modification correlated perhaps with chromosomal changes; and the second was "a neurogenic reflex" beginning from the 4-5-6-anterior dorsal branches and affecting the hypothalamus, thus stimulating the anterior pituitary to secrete prolactin. They believed that secretion of prolactin accounted for the gynecomastia.

Lachapele *et al.* (9), on the other hand, invoked three possibilities: 1) there may be decreased androgenic secretion (evident in his patient) with alteration of the androgen/estrogen ratio, due perhaps to general debilitation; 2) there may be a local modification due to an intrathoracic nervous stimulus—though this theory does not appear to be acceptable to the author himself; or 3) there may be a pituitary alteration due to an unknown pulmonary stimulus.

Podestá *et al.* (10) agreed with the last hypothesis. Unfortunately

Lachapele and associates did not present any data concerning the levels of hypophyseal hormone secretion to uphold the hypotheses they offered. Only Huguenin *et al.*, in 2 autopsy cases, found a pituitary anatomically and histologically normal. This obviously does not exclude the presence of some functional changes without any evident morphologic alteration.

Aubry *et al.* (24), reviewing all the endocrine disturbances occurring in association with pulmonary tuberculosis, suggested that these changes may be due to three different factors: 1) stress reaction, which may affect the pituitary gland; 2) toxins or auto-antigens, due to the local lung damage, which affect the infundibulum-tuber cinereum area, either organically or functionally; and 3) localization of tubercles in the hypothalamic-pituitary region.

In the cases of tuberculosis treated by INH, Guinet *et al.* (14) attributed the gynecomastia to the drug, basing observations on the previous work of Besta *et al.* (quoted by Guinet), who found alterations in the adrenal glands after treatment with INH, as manifested by an increase and then a decrease of 17-ketosteroid excretion.

On the other hand, Molina *et al.* (15) considered the possibility that the gynecomastia is due to the acid-fast infection itself, and suggested that a factor, beginning in the mediastinal-pulmonary area, is the medium for diencephalic-hypophyseal stimulation. They considered their theory confirmed when they found abnormalities in ovulation in animals with pulmonary tuberculosis.

Bongiovanni and Eberlein (20) in their study of newborn infants with pulmonary infection attributed the gynecomastia to the presence of maternal circulating hormones.

Villiaumey (21) considered the possibility that a peripheral nerve lesion was the stimulus for diencephalic-pituitary activity. In fact he stated that an intercostal nerve lesion, without evident pulmonary parenchymal involvement, may produce gynecomastia.

It appears that most authors agree in principle that in lung diseases there is an unknown stimulus which affects the diencephalon-pituitary region, with subsequent stimulation of gonadal or adrenal estrogens, resulting in stimulation of the breasts. There are, however, many obscure factors which must be elucidated.

What is the pulmonary stimulus? Is it endocrine in nature, as suggested by del Castillo, or is it toxic, allergic, or a general stress-reaction, as reported by Aubry? Perhaps it is merely due to neurogenic stimuli, as proposed by Vanni and Secchi and by Villiaumey. Valid studies are necessary before the mechanism can be definitely established.

In the present report, the pulmonary infection, though of extremely mild degree, certainly appeared to be the precipitating factor in the ensuing

breast stimulation. The repeated occurrence of the breast enlargement is unusual and fascinating, and implies that the stimulus was of a temporary nature.

The second point to be clarified is the nature of the pituitary hormone secreted under the influence of the unknown stimulus. It seems improbable that this can be the luteinizing hormone, in view of the low values for urinary 17-ketosteroids often found in male patients with gynecomastia. It also seems improbable that prolactin alone is the hormone, in view of the fact that prolactin by itself does not appear to be able to affect development of the breasts unless the mammary tissue has been previously primed with estrogens, either directly or by release of a pituitary mammotropic factor. Follicle-stimulating hormone (FSH) can indirectly induce gynecomastia, since this tropic hormone stimulates the ovaries to produce estrogens, which may accelerate mammary development. On the other hand, there are no data to show that FSH in the male (normally gametogenic) can cause increased hormone (estrogen or androgen) secretion by the testes.

In our patient the increased gonadotropin output correlated well with the appearance of breast development, as well as with the changes in the vaginal smear. Conversely, when the breasts and vaginal cells became atrophic, the urinary gonadotropin titer was below measurable levels by the mouse uterine-weight assay. Such negative gonadotropin titers are normal in the prepubertal child. Although this determination is a bioassay with a wide range of normal values in the adult, we have never found measurable urinary gonadotropin in a normal child prior to puberty. The vaginal cytology indicated increased endogenous estrogen levels sufficient to produce changes in the secondary sex characteristics. However, it may well be that other unknown pituitary hormones account in part for the breast hypertrophy, and that the elaboration of estrogen is secondary to these substances of hypophyseal origin.

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# LEYDIG-CELL TUMOR OF THE OVARY: CASE REPORT AND REVIEW

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ABSTRACT

A case of Leydig-cell tumor of the ovary in a 68-year-old woman is presented. Several hormone studies were made, but they yielded no evidence of increased secretion of androgen. Thirteen previously reported cases of ovarian Leydig-cell tumor are reviewed, and the conspicuous lack of increased male hormone activity in most instances is discussed.

VIRILIZATION in the adult female may be the result of endocrine imbalance from a variety of causes. Since the description by Berger in 1942 (1), Leydig-cell tumor of the ovary has been recognized as one of the conditions giving rise to the development of male characteristics in women. Twelve additional cases have appeared in the medical literature (2–13) since Berger's description. This neoplasm is believed to originate from undifferentiated cells in the hilum of the ovary. The precise hormonal alterations by which the induced masculinization can be explained have not been clearly elucidated.

In the following case of this relatively rare tumor, extensive hormonal studies were made.

## CASE REPORT

*P.K.* (#89015) a 68-year-old white female, was admitted to the surgical service of Montefiore Hospital on April 25, 1957, because of acute abdominal pain of forty-eight hours' duration. An exploratory operation several hours later revealed diverticulitis with perforation and generalized peritonitis. A colostomy was performed, and the postoperative course was uneventful. At the time of surgery it was noted that the patient presented an extremely masculine appearance (Fig. 1), so she was transferred to the medical service for further evaluation.

The patient had noted a receding hair line and an increase of facial and body hair for at least three years. Menarche had occurred in her early teens and she had continued to

Received May 26, 1960.

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menstruate regularly every three to four weeks until a spontaneous menopause at age 53. She had been married forty years without becoming pregnant, though contraceptive procedures had never been employed. Other pertinent history included known hypertension for at least twenty years.

*Physical examination* revealed a well developed woman with striking hirsutism of the face, arms, legs and trunk. There was a markedly receding hair line and spotty alopecia. The pubic escutcheon was primarily female in type. The voice was in the female register. The eyes were slightly prominent, but otherwise normal. The breasts were full and not atrophic. The heart was slightly enlarged to the left of the midline. Blood pressure was 180/90 mm. Hg; and the ventricular rate was 80 per minute and regular. A functioning

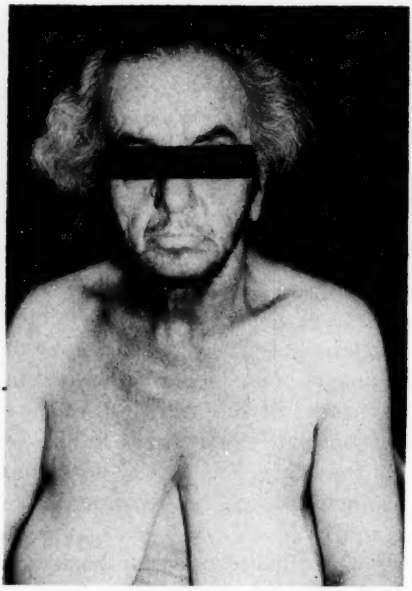

FIG. 1. Masculine appearance of Patient P. K., a 68-year-old woman.

colostomy opening was present in the right lower quadrant of the abdomen. The clitoris was enlarged to at least twice normal size. The pelvis was filled with a hard irregular mass which was located predominantly on the right side. It was impossible to differentiate uterus or adnexae from the mass.

*Laboratory data.* Random analyses of urine showed good concentration and the findings were essentially normal except for an occasional trace of albumin. The hematocrit was 40 per cent. The leukocyte count was 7400 per cu. mm., and the differential count was normal. The fasting eosinophil count was 120 per cu. mm. The level of blood urea nitrogen was 20 mg., fasting blood sugar 140 mg., creatinine 1.2 mg., and cholesterol 239 mg. per 100 ml. The levels of serum chloride, sodium, potassium, calcium, phosphorus, carbon dioxide and total protein were normal, as was the albumin/globulin ratio. The electrocardiogram revealed normal sinus rhythm, occasional premature ventricular contractions and an enlarged left ventricle. X-ray films of the chest showed nothing abnormal except for rounding of the left ventricle; x-ray films of the skull showed marked hyperostosis frontalis interna, some hyperostosis of the temporal lobe region, and extensive calcification of the falx cerebri; the sella turcica was normal. An intravenous

pyelogram was normal. A barium-enema x-ray examination showed extensive diverticulitis. The results of hormone studies are listed in Table 1. Urine chromatography was performed as follows: urine was collected over a period of three days and was then incubated with β-glucuronidase; this was followed by continuous extraction with ether at pH 1. The neutral extract was chromatographed according to the method of Lakshmanan and Lieberman (13). Only two Zimmermann peaks indicating less than the equivalent of 0.5 mg. of 17-ketosteroids were obtained. By their positions in the chromatograms these compounds were identified as androsterone and etiocholanolone. No further identification was attempted. A Papanicolaou vaginal smear was read as "atrophic, Class I, no tumor cells present."

*Hospital course.* The patient recovered uneventfully from the initial operation. On August 8, 1957, she returned to the operating room for a sigmoid resection and removal of the ovarian mass. Following this operation a wound infection developed, which was refractory to antibiotics. This inflammatory process spread to the genito-urinary tract and resulted in progressive renal failure. The patient died on September 11, 1957. Permission for autopsy was not obtained.

*Pathologic findings.* The tissue removed at operation consisted of a leiomyoma, a resected sigmoid and a right ovarian tumor. The ovarian tumor was described as follows: The specimen consisted of a firm, rubbery, nodular mass measuring 4.8×3.9×2.0 cm. in greatest dimensions. The surface was gray-white and had the appearance of normal ovarian tissue. A glistening serous membrane completely invested the mass, with the exception of one pole which represented the attachment of the tubalovarian ligament. On cut section a well demarcated nodule was seen, which measured 2.3 cm. in greatest diameter. It was solid, firm and grayish white. The surrounding ovarian parenchyma had a normal variegated appearance.

Microscopic examination showed that the ovarian tumor consisted of a large amount of pale eosinophilic homogeneous stroma in which were varying-sized islands of cells, largely arranged in small clusters (Fig. 2). In places, they tended to form cords and even rosettes. The nuclei of these cells were, in general, regular and vesicular; nucleoli were present. Several of the cells were binucleate and even trinucleate, but mitotic figures were exceedingly rare. The cytoplasm of the cells was granular and deeply eosinophilic (Fig. 3). In addition, some cells contained a tan-brown pigment. This pigment was lying free in the stroma in some areas. No inclusion bodies or crystalline materials were seen. The stroma was palely eosinophilic and largely homogeneous; it contained a few scattered fibroblasts and some isolated polygonal cells which resembled those found in the cellular islands. There were no areas of degeneration and necrosis, and there was no evidence of a capsule. An adjacent rim of ovarian tissue was composed of normal-appearing stroma with occasional corpora albicantia. There was no vascular invasion. The diagnosis was "Leydig-cell tumor of the ovary," a diagnosis which was confirmed by the Registry of Ovarian Tumors in Baltimore, Maryland.

## DISCUSSION

In 1923 Berger described certain "sympatico cells" in the hilum of the ovary which were capable of producing androgen. A neoplasm derived from cells with such hormonal function would therefore be expected to result in masculinization. The 13 reported cases corroborate this hypothesis. In addition, several investigators have described virilization associated with hyperplasia of ovarian Leydig cells (14–17). Despite the extensive hormonal studies performed in the case presented here (including determination of

TABLE 1. SUMMARY OF PATHOLOGIC, HORMONAL AND CLINICAL FINDINGS IN 14 REPORTED LEYDIG-CELL TUMORS

| Reference | Age of patient (yrs.) | Pathologic findings | Hormone studies | | | | | Clinical data |
|---|---|---|---|---|---|---|---|---|
| | | | 17-Ketosteroid excretion (mg./24 hrs.) | FSH excretion (m.u./24 hrs.) | Pregnanediol & pregnanetriol excretion | Estrogen levels | Androgen excretion | |
| Sachs & Spiro (2) | 47 | Right ovarian tumor; Ca. of lung | 7.3–8.7; beta fraction, 0.16 | Negative titer | — | — | — | Amenorrhea for 7 yrs.; hirsutism; alopecia for 4 yrs. |
| Pedersen & Hamburger (3) | 65 | Right ovarian tumor | 11.7–21.5; beta fraction <17 (normal, up to 7.5) | <50 | Pregnanediol 0 | 20–200 m.u./24 hrs. in urine (normal) | 72–145 I.U./24 hrs. | Amenorrhea for 33 yrs.; hirsutism; alopecia; deep voice; enlarged clitoris. |
| Waugh *et al.* (4) | 46 | Left ovarian tumor; large left adrenal gland | 16–22.4* | — | — | Vaginal smear; no estrogen effect | — | Amenorrhea for 11 yrs.; hirsutism for 6 yrs.; alopecia. |
| Segaloff *et al.* (5) | 39 | Right ovarian tumor | 15.6 | — | — | — | — | Amenorrhea and hirsutism for 6 yrs. |
| Sternberg (6) | 86 | Bilateral ovarian tumors | 6.9 | — | — | — | — | Hirsutism and enlarged clitoris for 32 yrs. |
| Sternberg (6) | 64 | Right ovarian tumor | 7.5 | — | — | — | — | Hirsutism and deep voice for 2 yrs. |
| Stolk (7) | 72 | Ovarian tumor | 8.4 | — | — | — | — | Vaginal bleeding; endometrial polyposis with cystic hyperplasia |
| Israel & Mutch (8) | 54 | Right ovarian tumor | 18–25 (normal, up to 25) | — | — | — | — | Amenorrhea and alopecia for 11 yrs. |
| Berkheiser (10) | 50 | Right ovarian tumor | — | — | — | — | — | Hirsutism for 25 yrs.; alopecia for 8 yrs.; enlarged clitoris; deep voice. |
| Berger (1) | 50 | Mesovarian tumor | — | — | — | — | — | Enlarged clitoris for 18 yrs.; hirsutism; male escutcheon; menstruated until hysterectomy. |
| Young (9) | 27 | Right ovarian tumor | — | — | — | — | — | Pregnant at time of surgery; menses had been irregular; progressive enlargement of clitoris prior to surgery; hirsutism; male escutcheon. |
| CPC, New England J. Med. (11) | 60 | Left ovarian tumor | 4.6; 5.2 | — | — | 22 μg./24 hrs. in urine (normal) | — | Hirsutism for 2–3 yrs.; enlarged clitoris. |
| Scully (12) | 64 | Left ovarian tumor | | | | | | Amenorrhea for 20 yrs., then bloody vaginal discharge; small focus of adenocarcinoma of uterus at time of surgery. |
| German *et al.* (present case) | 68 | Right ovarian tumor | 4.5<br>Blood corticoids, 10 μg./100 ml.; with ACTH test —rise of 16–45 μg./100 ml. | <16 m.u. | Trace of both | Vaginal smear; atrophic cells | | Amenorrhea for 15 yrs. (natural menopause); hirsutism for 3–4 yrs.; alopecia; enlarged clitoris. |

urinary pregnanediol and pregnanetriol, and the use of chromatographic methods), no evidence of increased androgen activity was found. It appears unlikely that this was due to impaired renal or hepatic function, since the intravenous pyelogram, blood urea nitrogen and creatinine levels, urinalysis findings, and serum protein concentration were normal at the time of the investigation.

In reviewing the previous reports (Table 1), it is interesting that there was evidence of high androgen activity in only 2 cases (3–4) of 10 in which

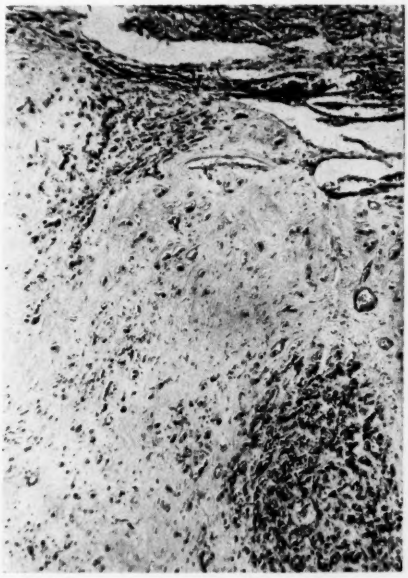

FIG. 2. Edge of the ovarian tumor, showing the abundant dense stroma. (H & E, reduced from ×70.)

hormonal determinations were performed. In one of these (4), there was an enlarged left adrenal gland, which might explain the elevated 17-ketosteroids. Recently Anliker *et al.* (18) and Wiest *et al.* (19) have isolated testosterone, androsterone and androstene-3,17-dione from arrhenoblastomas. However, elevation of total 17-ketosteroids has been infrequently reported in this type of neoplasm.

In the present case, several possible explanations should be considered: 1) the tumor may have been secreting small amounts of testosterone or androsterone which, over a long period of time, were sufficient to virilize the patient though they were insufficient to be detected by our techniques, 2) the substance causing the virilization may not have been excreted as one of the urinary metabolites that were measured, and 3) the tumor may no longer have been active, though this is an improbable explana-

tion in view of the activity strongly suggested by the patient's need to shave every other day and the increasing alopecia while in the hospital. In our patient as well as in a few others, gonadotropin excretion was determined; the titers were lower than would be expected in a postmenopausal woman. There was no evidence of hypopituitarism in our patient to explain this, and the atrophic condition of the cells in the vaginal smear ruled out estrogenic activity of any consequence.

Therefore, we can only conclude that the substance causing the virilization of the patient also accounted for the low excretion of gonadotropin.

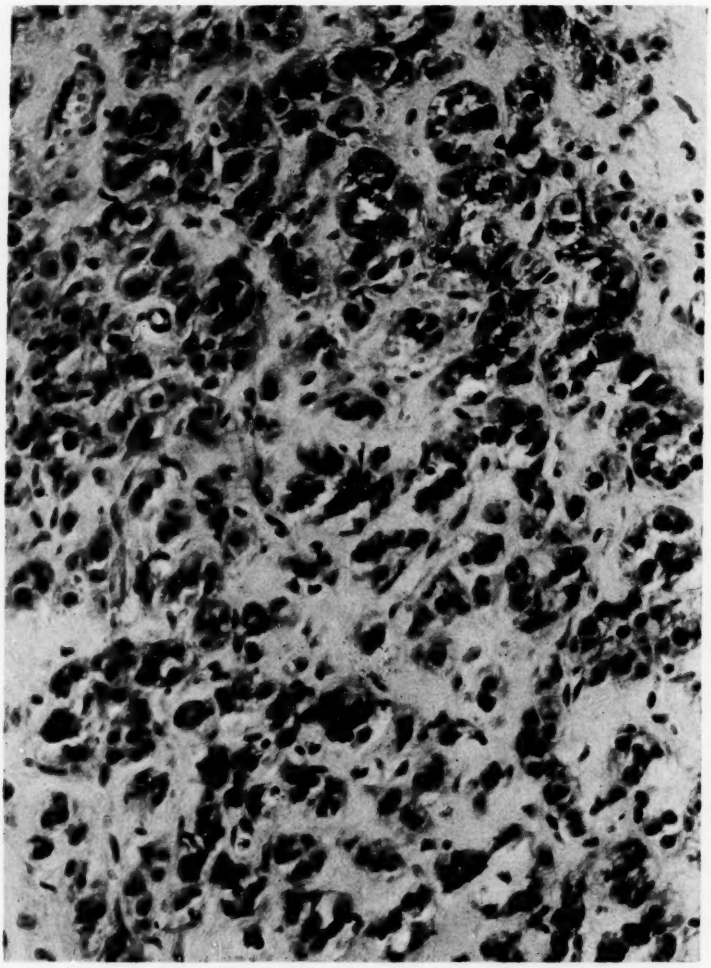

FIG. 3. Cellular pattern in the ovarian tumor, showing the regularity of the cells and their abundant granular cytoplasm. (H & E×400.)

*Acknowledgments*

The authors wish to express their appreciation to Dr. A. M. Bongiovanni and Dr. W. R. Eberlein, Children's Hospital, Philadelphia, Pa., for performing the assays of pregnanediol and pregnanetriol, and to Mr. Antol Hirskovitz for preparing the photographs.

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# Letters to the Editor

## ESTIMATION OF TESTOSTERONE IN HUMAN PLASMA*

To the Editor:

Testosterone has been detected in spermatic vein blood and in human peripheral blood after stimulation with human chorionic gonadotropin but not in the peripheral blood of untreated subjects (1). This report describes a method for measuring testosterone in blood. It is based on the fact that testosterone can be enzymatically converted to estradiol-17$\beta$ and estrone (2) and these latter compounds can be estimated in extremely minute quantities by a fluorometric technic (3, 4). Testosterone in concentrations of 0.1 $\mu$g. per 100 ml. of human plasma has been estimated by this method, which involved the following procedures:

### METHOD

1) A 50-ml. sample of plasma was subjected to extraction four times with equal volumes of ether:chloroform, 3:1. The combined extracts were washed with 1*N* NaOH to remove acidic and phenolic compounds, and finally washed with saturated $NaHCO_3$ and water. The neutral extracts were dried over $Na_2SO_4$ and concentrated to dryness.

2) The dried extract, containing the neutral compounds, was dissolved in 70 per cent methanol and stored in a deep-freeze overnight. When a precipitate formed, the mixture was rapidly centrifuged and the clear supernatant liquid was partitioned against petroleum ether (1).

3) The methanol was removed by distillation under reduced pressure, and the resulting aqueous residue was subjected to extraction three times with equal volumes of benzene. The combined benzene extracts were washed once with water, dried over $Na_2SO_4$, and concentrated to dryness. The dried residue was chromatographed in the ligroin-propylene glycol system (5) in parallel with a testosterone standard for seventy-two hours at 27° C.

4) The testosterone zone was located by viewing in short ultraviolet light. The material contained in this testosterone zone was incubated with a placental enzyme preparation[1] (2) in the presence of 0.5 $\mu$M of TPN, 5 $\mu$M of glucose-6-phosphate, 0.5 Kornberg

* Supported in part by American Cancer Society Institutional Grant No. In-56, and Grant No. A-2672 of the U. S. Public Health Service.

[1] The enzyme was prepared according to the method of Ryan (2). Each pellet, obtained after 105,000 g centrifugation, was resuspended in 3.0 ml. of phosphate buffer (0.1*M*, pH 7.2), and 0.5 ml. of the suspension was used as the source of enzyme. The activity of the enzyme preparation was checked with each run by incubation of testosterone-4-$C^{14}$ and determining the extent of conversion to estradiol-17$\beta$ by both counting and fluorometry. The placental preparation was found to be stable after several months' storage in the deep-freeze.

unit glucose-6-phosphate dehydrogenase, 50 $\mu$M of phosphate buffer at pH 7.2, and 0.154 M of KCl to make a final volume of 1.0 ml. The incubations were carried out for one and a half hours at 37° C. in air in a Dubnoff Metabolic Shaker.

5) The reaction was stopped by adding 10 volumes of ethanol. Insoluble material was removed by centrifugation and the precipitate was washed with half the original volume of ethanol. The ethanol supernatants were combined and evaporated to dryness. The dried residues were moistened with a little ethanol and then taken up in 10 ml. of benzene. The benzene solution underwent extraction once with 10 ml. and twice with 5 ml. of 1*N* NaOH. The pooled alkali extracts were placed in an ice bath and the pH adjusted to about 8.0 with concentrated HCl; extraction was carried out with three 10-ml. portions of benzene. The benzene extracts were washed successively with 3.0 ml. of 30 per cent $H_2SO_4$, water, saturated $NaHCO_3$, and again with water to neutrality. The benzene extracts were dried over $Na_2SO_4$ and then evaporated to dryness in a stream of nitrogen. The dried residues were chromatographed on paper in the benzene : methanol system (6) along with standard estradiol-17$\beta$ and estrone. The respective zones were eluted and quantitatively estimated fluorometrically (4, 6). In model experiments, testosterone-4-$C^{14}$ was added to plasma, converted to estrogenic material enzymatically, and the conversion products were localized on paper by scanning. The concentration was estimated both by counting and by fluorometry.

6) To control the over-all recovery and to assist in detecting the zones on paper, 0.01 $\mu$g. or less of testosterone-$C^{14}$ or testosterone-$H^3$ with a high specific activity may be added to the 50-ml. sample of plasma.

## RESULTS

### *Recovery experiments*

A 0.3-$\mu$g. portion of testosterone-4-$C^{14}$ (9000 cpm) was added to 50 ml. of plasma and subjected to the described procedure. In four experiments the major conversion product was estradiol-17$\beta$ and the over-all mean recovery was 50 per cent (as estradiol-17$\beta$) on the basis of the $C^{14}$ count, and 55 per cent when the product was measured fluorometrically (Table 1).

### *Estimation of testosterone in normal plasma*

Five plasma samples, including 3 from normal men and 2 from normal women, were analyzed for testosterone content. The values in 2 of the men were 0.2 $\mu$g. and 0.4 $\mu$g. per 100 ml. of plasma, and in the other man the value was less than 0.1 $\mu$g. In the 2 women, values of 0.1 $\mu$g. and less than 0.1 $\mu$g. per 100 ml. of plasma were found (Table 2).

TABLE 1. RECOVERY OF ADDED TESTOSTERONE-4-$C^{14}$ FROM HUMAN PLASMA

| Plasma sample | Percentage recovery as estradiol-17$\beta$ after chromatography based on | |
|---|---|---|
| | cpm | fluorometry |
| I | 55 | 63 |
| 2 | 47 | 58 |
| 3 | 55 | 58 |
| 4 | 44 | 50 |

TABLE 2. CONCENTRATION OF TESTOSTERONE IN HUMAN PLASMA

| Subject | | | Testosterone in plasma (μg./100 ml.) |
|---|---|---|---|
| Case No. | Diagnosis | Sex & Age* | |
| 1 | Normal | M 40 | <0.1 |
| 2 | Normal | M 35 | 0.2 |
| 3 | Normal | M 28 | 0.4 |
| 4 | Normal | F 35 | 0.1 |
| 5 | Normal | F 22 | <0.1 |
| 6 | Adrenal adenoma | F 27 | 1.3 |
| 7 | Ovarian hilus-cell tumor | F 71 | 2.0 |

* Because of the limited number of samples, no significance should be attached at present to a possible relationship between age and testosterone levels in plasma.

Plasma samples from 2 female patients with virilizing syndromes were also studied. Patient No. 6 (Table 2) was suffering from an adrenal adenoma and excreted a gram or more of 17-ketosteroids daily.[2] Patient No. 7 (Table 2) had a hilus-cell tumor of the ovary with exceedingly low urinary 17-ketosteroids.[3] In Patient No. 6 the plasma level of testosterone was 1.3 μg. per 100 ml. and in Patient No. 7 it was 2.0 μg. per 100 ml.[4]

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*July 13, 1960*

*Acknowledgment*

The authors are most grateful to Dr. F. Sciarra and Miss M. Stylianou for their devoted assistance.

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[2] This sample was made available to us by the kindness of Dr. Irene T. Kline and Dr. Richmond Smith, of the Henry Ford Hospital, Detroit, Michigan.

[3] We are grateful to Dr. Thomas B. Connor, of the University of Maryland School of Medicine, for this sample.

[4] This plasma sample represented a pool obtained before and during a 6-hour infusion of 25 units of ACTH.

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## HYPERCALCEMIA AS A COMPLICATION OF VITAMIN D THERAPY IN POSTMENOPAUSAL OSTEOPOROSIS

TO THE EDITOR:

Osteoporosis in the postmenopausal female is without doubt the most common variety of bone disease in the United States today. In a recent study of 218 ambulatory women past 45 years of age seen in an out-patient department for complaints unrelated to bone disease, 29 per cent had osteoporosis of such a degree as to be unquestionably apparent in skeletal roentgenograms (1).

When osteoporosis is discovered in the postmenopausal female, routine management often consists of cyclic estrogen and androgen therapy, and the daily administration of vitamin D in small doses. In the following 2 patients, this regimen ultimately was complicated by the development of hypercalcemia:

### CASE REPORTS

*Case 1*

*V. H.*, a 60-year-old female, was first seen in 1955 with the chief complaint of low back pain. Roentgenograms showed marked osteoporosis and compression fractures of the bodies of the vertebrae. At that time she received cyclic estrogen and androgen therapy, and 3 glasses of milk and 50,000 units of vitamin D daily.

With this treatment she experienced relief of back pain and progressed satisfactorily until 1959, when she began to have morning headaches. Mild hypertension was discovered on examination. This was soon followed by the precipitous onset of anorexia, nausea, vomiting and severe constant headache, for which she was admitted to the hospital.

At that time (1959) the blood pressure was 210/120 mm. Hg. The serum calcium level was 17.5 mg. per 100 ml., and phosphorus 3.3 mg. per 100 ml. on two separate occasions. Treatment was promptly started with di-sodium versenate and hydrocortisone. Within a few days, the serum calcium concentration had returned to a near-normal level. At the time of discharge from the hospital, one month following admission, the concentration of serum calcium was 10.4 mg. per 100 ml., and of phosphorus 2.9 mg. per 100 ml. The blood pressure was within the normal range.

*Case 2*

*D. H.*, an 83-year-old female, was first seen in March 1958, for routine care of diabetes. At that time a chest roentgenogram revealed osteoporosis. Treatment was started with cyclic estrogen and androgen therapy, and 3 glasses of milk and 50,000 units of vitamin D daily. She was followed as an out-patient until November 1959, when she was admitted for regulation of diabetes and evaluation of general malaise and a tendency to stagger.

On the day of admission her serum calcium level was 12.3 mg. per 100 ml., and phosphorus 3.7 mg. per 100 ml. She was given a low-calcium diet and vitamin D was discontinued. The next day the serum calcium level was 12.0 mg. per 100 ml., and phosphorus 3.2 mg. per 100 ml. At the time of discharge, two weeks following admission, the serum calcium level was 10.7 mg. per 100 ml., and phosphorus 3.1 mg. per 100 ml. Subsequent values were all within normal limits.

In both cases, additional studies in an attempt to uncover other possible causes for the loss of skeletal bone or for the abnormal susceptibility to small doses of vitamin D yielded negative results. Both patients repeatedly denied taking more than the prescribed dose of 50,000 units of vitamin D daily.

## DISCUSSION

It appears to be well established that the administration of estrogens, with or without the addition of androgens, favorably affects the clinical course of postmenopausal osteoporosis and brings about a definite and prolonged positive calcium balance in the great majority of cases (2, 3).

The effect of increased calcium intake and administration of vitamin D in this condition is less well defined. The classic concept of the pathogenesis of osteoporosis regards the failure of maintenance of the normal protein matrix of bone as the primary defect, and the subsequent loss of mineral as only a secondary manifestation. In the light of such a concept, the administration of an excess of calcium or of vitamin D might seem unsound. There is, however, evidence to suggest that such therapy has a rational basis.

Shorr and Carter (4) demonstrated that the addition of excess calcium or vitamin D to estrogen therapy results in a further increase in the positive calcium balance. Vinther-Paulsen (5) found that the intake of calcium and phosphorus in a group of patients with osteoporosis was much lower than in

a control group. With radioactive calcium techniques, Whedon (6) showed that calcium deposition is normal in osteoporosis. He also demonstrated a positive relationship between the amount of calcium ingested in the diet and the amount of calcium retained in the body, through a wide range of intakes.

Although the administration of vitamin D may favorably influence postmenopausal osteoporosis, particularly in the presence of a low-calcium intake, the 2 cases presented in this report demonstrate that it is not always innocuous and that dosages as small as 50,000 units daily may occasionally lead to most severe consequences. Since the apparently beneficial provision of adequate calcium can be accomplished by dietary means, vitamin D is best omitted from the therapeutic program for postmenopausal osteoporosis.

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# IS THYROXINE BINDING BY PRE-ALBUMIN OF PHYSIOLOGIC IMPORTANCE?

To the Editor:

It is well known that most of the circulating thyroxine ($T_4$) is associated with a small protein fraction (TBG) migrating between the $\alpha_1$ and $\alpha_2$ globulins (1). A small proportion is bound to serum albumin. Ingbar (2) showed in 1958 that the proteins in the pre-albumin area have a strong affinity for thyroxine. He used Tris-maleate buffer of pH 8.6 for electrophoretic separation. In this system about 30 per cent of the thyroxine was found attached to pre-albumin. Most previous studies had been carried out with veronal buffer (3). Ingbar (4) has suggested that veronal interacts with pre-albumin and thus inhibits its ability to bind $T_4$.

Some of the possible physiologic and clinical implications of this demonstrated $T_4$-binding to pre-albumin have been discussed by Ingbar (4). He also debated the question of whether or not this phenomenon might be an electrophoretic artefact induced by Tris-maleate buffer, but found no basis for such an assumption.

Some doubt has, however, still existed concerning this problem. Studies by one of the authors (5) suggest that $T_4$ and triiodothyronine ($T_3$) are bound to the same binding sites in plasma at pH 7.4. Since $T_3$ is not bound to pre-albumin, this is another indication that the binding of $T_4$ to this protein might be an artefact.

Hamolsky *et al.* (6) found that the proportions of $T_4$ bound to TBG and to pre-albumin could vary considerably, dependent upon the experimental conditions.

This problem consequently led us to study the paper-electrophoretic distribution of $T_4$-$I^{131}$ in serum at pH levels near the near physiologic values.

## METHOD

Serum containing 5 $\mu$g. of *l*-$T_4$-$I^{131}$ per 100 ml. was electrophoresed in the cold room at 240 volts (8 volt/cm.) for eighteen hours in 0.1 *M* phosphate buffer at pH 7.6 (Whatman No. 1 paper). The distribution curve of the $T_4$-$I^{131}$ was obtained by a strip-counter (Nuclear-Chicago Corp.), after which the paper was stained to locate the albumin spot.

Figure 1 shows no $T_4$-$I^{131}$ ahead of the albumin zone (upper curve). This might be due to poor separation of the pre-albumin from the other proteins. Pre-albumin cannot ordinarily be visualized on the paper strip. The preceding possibility was therefore ruled out by electrophoresing the same serum with $I^{131}$-labeled tetraiodothyroacetic acid (Tetrac) in the same experiment. This thyroxine analogue is practically exclusively bound to pre-albumin (4). The pattern observed in the lower curve of Figure 1 suggested that pre-albumin was well separated from the albumin.

The possibility that only Tetrac pre-albumin should migrate ahead of the albumin seems unlikely. The association of Tetrac with pre-albumin gives the latter a higher negative charge. Paper electrophoretic experiments at pH 8.6 (Tris buffer) showed, however, no significant difference in the distances of the thyroxine pre-albumin and the

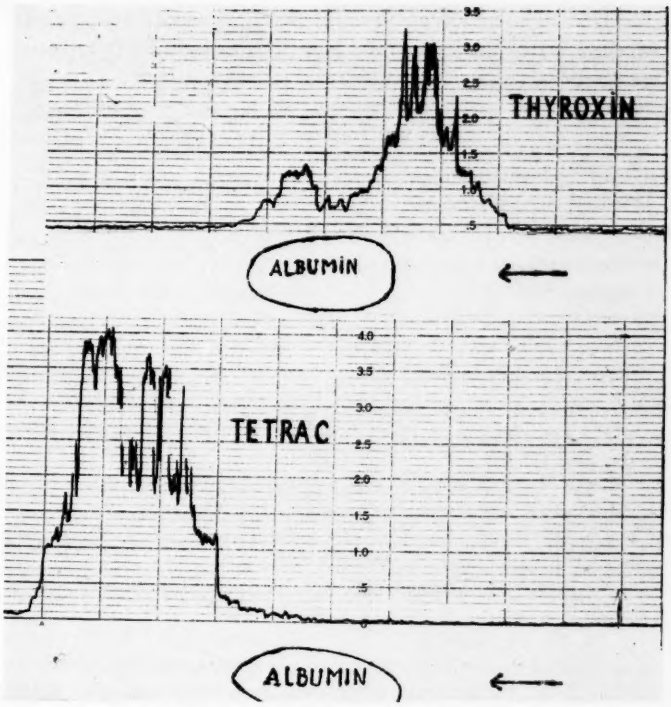


FIG. 1. Distribution of radioactivity on paper electrophoretic strips (normal human serum with added $I^{131}$-labeled thyroxine and tetraiodothyroacetic acid).

Tetrac pre-albumin peaks from the origin. A similar distribution pattern was obtained by using Tris buffer (pH 7.6) instead of phosphate buffer.

## DISCUSSION

The foregoing experiments suggest that the pre-albumin binding of $T_4$ is negligible at physiologic pH values. In this connection the following observations may be of interest (7). By a dialysis procedure it was shown that a shift of the pH into the alkaline range results in a decrease of the over-all binding of $T_4$ to the plasma proteins. The binding to serum albumin, however, increased in association with the increase in pH, and this might possibly also be true of pre-albumin. These results could thus be explained by a decrease in binding to TBG at this pH, combined with a possible increase in the binding capacity of pre-albumin itself. The pre-albumin binding of $T_4$ at pH 8.6 seems not to be dependent upon any specific buffer effect, since we found approximately the same amount of $T_4$-$I^{131}$ in the pre-albumin area whether we used Tris or borate buffer.

Pre-albumin seems to have a distinct affinity for thyroxine (2, 4). At

physiologic pH values, however, most of the $T_4$ is associated with the much stronger binding TBG, and the pre-albumin binding of the hormone seems without major importance.

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## EXTRA-ADRENAL INFLUENCE OF CORTICOTROPIN (ACTH) ON CORTISOL METABOLISM

To the Editor:

Dougherty *et al.* (1) studied the influence of corticotropin (ACTH) on the biologic half-life of radioactive cortisol in 2 patients, one without endocrine disorders and the other suffering from Addison's disease. Similar experiments in adrenalectomized mice as well as *in vitro* work by the same authors seem to indicate an extra-adrenal effect of ACTH. In the present study we have confirmed and extended their observations by use of non-radioactive cortisol.

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## METHODS

In normal subjects and in 2 patients with adrenal insufficiency the rate of disappearance of *exogenous* cortisol was determined: (*a*) under basal conditions, *i.e.*, without previous administration of ACTH, and (*b*) after an infusion of ACTH over a period of eight hours (50 I.U. in 500 ml. of physiologic saline, from 8:00 a.m. till 4:00 p.m.). Cortisol (1 mg. per Kg. of body weight) was infused as prescribed by Brown *et al.* (2). Blood samples were drawn at approximately two, three, five, six and seven hours after the beginning of the cortisol infusion.

Disappearance curves of *endogenous* cortisol were obtained by taking blood at different time intervals during the seven-hour period following the infusion of ACTH, but without the injection of additional cortisol.

Plasma corticoid levels were determined by a fluorometric technic reported in detail elsewhere (3). In 14 patients the colorimetric method described by Peterson *et al.* (4) was used on the same blood samples; similar values were found with the two methods.

Cortisol disappearance rates were calculated using the method of the least squares and assuming that the exponential equation

$$C = C_o e^{-kt} \qquad \text{(I)}$$

$C_o$ = plasma corticoid concentration at time zero
$C$ = plasma corticoid concentration at time $t$
$k$ = disappearance constant

described the data both without and with ACTH infusion. Almost identical results were obtained using an analog computer (5) and the equation

$$C = (C_o - C_{eq})e^{-kt} + C_{eq} \qquad \text{(II)}$$

$C_{eq}$ = equilibration concentration of plasma corticoids.

## RESULTS

In Table 1 the data obtained after ACTH infusion have been compared with the basal values at about the same time of the day. The post-ACTH disappearance rate of *exogenous* cortisol given at 4:00 p.m. was definitely lower ($k = 0.198$) than the rate under basal conditions at 8:00 p.m. Disappearance of the cortisol secreted by the subjects themselves (*endogenous*) after ACTH infusion was even slower ($k = 0.143$). In the 2 patients with adrenal insufficiency, the mean removal rate was 0.321 without ACTH and 0.180 with ACTH—a difference similar to that noted in the exogenous cortisol group.

One could speculate that cortisol production by the adrenal continued after the discontinuance of the ACTH infusion and thus slowed down the post-ACTH disappearance of cortisol. The fact that a graph of the logarithms of the plasma corticoid concentrations plotted against time appears to be a straight line, is not sufficient proof of the contrary (6). However, the results obtained in the cases of Addison's disease exclude this hypothesis.

Dougherty *et al.* (1) believe their data indicate that ACTH inhibits the steroid-conjugating capacity of the liver, but the slower post-ACTH disappearance of cortisol could also be the result of interference by ACTH with the re-entry of free cortisol into the plasma compartment from the

TABLE 1. INFLUENCE OF ACTH ON THE DISAPPEARANCE RATE OF CORTISOL

| | Results | | | Subjects | | | |
|---|---|---|---|---|---|---|---|
| | CO* (μg./100 ml.) | Removal rate | Half-time (min.) | Male | Female | Total | Mean age (yrs.) |
| *Basal conditions:* Exogenous cortisol at 8 p.m. | 109 ±29** | 0.294 ±0.040* | 163 | 5 | 3 | 8 | 36 |
| *Post-ACTH:* Exogenous cortisol at 4 p.m. | 126 ±34 | 0.198 ±0.039 | 212 | 8 | 3 | 11 | 40 |
| *Post-ACTH:* Endogenous cortisol at 4 p.m. | 62 ±17 | 0.143 ±0.064 | 294 | 11 | 11 | 22 | 37 |

* Plasma corticoid concentration at time zero.
** Standard deviation.
Significance of differences of removal rates:
Basal 8 p.m. versus post-ACTH, exogenous; $P < 0.001$.
Basal 8 p.m. versus post-ACTH, endogenous; $P < 0.001$.

extravascular site (7). Furthermore, if the steroid-conjugating capacity of the liver should prove to be the mechanism of interference, corticoid levels as determined by the Peterson procedure (which include nonconjugated tetrahydrocortisone and tetrahydrocortisol) should be higher than those determined by the fluorometric method. Actually, closely similar plasma corticoid values were obtained with both methods, the colorimetric values being somewhat lower than those obtained by the fluorometric technic. Therefore it seems probable that, if the liver is involved, hydrogenation of the A ring, and not conjugating capacity is the mechanism of interference. As to the slower post-ACTH disappearance rate of *endogenous* cortisol compared to that of *exogenous* cortisol, no explanation is offered for the moment.

Visualizing the role of ACTH in cortisol catabolism without the additional positive influence on production of the hormone remains a complex problem. In any event, the extra-adrenal effects discussed here may be contributory in maintaining high, biologically active corticoid levels in several pathologic entities.

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# The Endocrine Society

## The 1961 Annual Meeting

The Forty-Third Annual Meeting of the Endocrine Society will be held in the Hotel Biltmore, New York, N. Y., Thursday, Friday and Saturday, *June 22, 23 and 24, 1961.*

The Scientific Sessions will be held from 9:00 a.m. to 5:00 p.m. daily and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 23, at 7:30 p.m., preceded by cocktails at 6:30 p.m.

All members are urged to make their hotel reservations immediately. The Biltmore will hold 300 bedrooms for members until May 1, 1961, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations early, directly with the hotel, advising them of time and date of arrival and departure. If you plan on remaining through June 26 for the American Medical Association and other meetings, it is imperative that your reservations be made from June 26 on, directly through the A. M. A. Housing Bureau. Make your reservations early and avoid disappointment. Hotel reservation card will be mailed to members shortly after January 1, 1961.

Final program, membership card and advance registration forms will be sent on May 1, 1961 to members whose current dues have been paid.

*Those wishing to present papers,* which will be strictly limited to ten minutes, should send an original and four copies of the title and abstract to the Vice-President, Dr. Roy Hertz, National Cancer Institute, Bethesda, Maryland, not later than *February 15, 1961.* It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately 200 abstracts submitted each year and compiling of the program from these involves a tremendous amount of time and effort on the part of the Program Committee.

THE COUNCIL REQUESTS THAT AUTHORS ADHERE STRICTLY TO THE FOLLOWING RULES WHEN SUBMITTING ABSTRACTS; OTHERWISE THEY CANNOT BE CONSIDERED:

1. IT IS ASSUMED THAT ABSTRACTS SUBMITTED FOR THIS PROGRAM HAVE NOT AND WILL NOT BE SUBMITTED ELSEWHERE.
2. Abstracts may not exceed 200 words, or equivalent space, exclusive of title. No footnotes or acknowledgments to sponsors can be published. References, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulae cannot be used.
3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed 15 words;
   Line 2. Author/s. The name of each nonmember-author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of nonmembers who are introduced, *i.e.*, who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . . )." The principal degree, *e.g.*, M.D., of each author should be written after his name.
   Line 3. Institution of origin and city in which institution is located.
4. Authors who wish receipt of their abstracts acknowledged should enclose self-addressed post card with the title of the abstract noted thereon.
5. Authors are also to enclose 3×5 inch filing cards, one for each author, giving his name and initials in the upper right-hand corner, and the complete title of the abstract with all the authors listed in the center of the card. There should be a second card enclosed listing the title and all authors, and the mailing address of the senior author. This is necessary for indexing. Senior authors will be notified of the acceptance of their papers for the final program.
6. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. The original and four copies should be forwarded to Dr. Hertz.
7. Abstracts should be letter perfect, since there will be no opportunity for proof reading by the authors.

## The 1961 Awards

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors. Endocrine Society awards are limited to citizens of the United States and Canada.

### FRED CONRAD KOCH AWARD

In 1957 a substantial legacy was bequeathed to the Society by the late

Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, Distinguished Service Professor of Physiological Chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is the highest honor of The Endocrine Society and is represented by a medal known as the Koch Medal of The Endocrine Society. The medal, as well as an honorarium of $3,500, is to be given annually to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada.

This Award was first presented in 1959, to Dr. W. W. Swingle and in 1960 was presented to Dr. Emil Witschi. The Koch Medal has replaced the Medal of The Endocrine Society which was established in 1954.

### The Ciba Award

The Ciba Award is given in recognition of the meritorious accomplishments of an investigator in the field of basic or clinical endocrinology who has not reached his thirty-sixth birthday before June 1 of the year in which the Award is presented. This Award was established in 1942 and first presented in 1944. The Award is presently in the amount of $2,500.

### The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

### The Upjohn Scholar of the Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. A grant has been made available through the generosity of the Upjohn Company, and will be awarded to an established investigator or teacher in the field of endocrinology who wishes to extend the opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually and will be granted on the basis of proposals submitted by the applicant. Such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

### Nominations

Nominations for the Fred Conrad Koch Award, the Ciba Award, and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than *October 15* each year.

Proposals for appointments as a Scholar of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by *October 15* each year.

## The American Goiter Association

The next Annual Meeting of the American Goiter Association will be held in the Warwick Hotel, Philadelphia, Pennsylvania, *May 3–6, 1961*. Arrangements are being made for a Clinical Day in the Philadelphia Hospitals on May 3. Scientific papers will be presented in the Warwick Hotel on May 4, 5 and 6. For further information address the Secretary, John C. McClintock, M.D., 702 Madison Avenue, Albany 8, N.Y.

## Symposium on Fundamental Cancer Research

The University of Texas M. D. Anderson Hospital and Tumor Institute announces the Fifteenth Annual Symposium on Fundamental Cancer Research—"The Molecular Basis of Neoplasia"—to be held *February 23–25, 1961*, in the M. D. Anderson Hospital Auditorium in Houston, Texas. Further information may be obtained from the Publications Department at The University of Texas M. D. Anderson Hospital and Tumor Institute Texas Medical Center, Houston 25, Texas.

## Training Program in Radiation Biology and Cancer Related Research

The Bowman Gray School of Medicine is beginning a training program in Radiation Biology and Cancer Related Research. One-, two-, and three-year traineeships are being offered to research-oriented people in the basic and clinical sciences, both at pre-doctoral and post-doctoral levels, and stipends will range from $1800.00 per annum up to $8,000.00 per annum.

This training program represents an excellent opportunity for young men interested in research to learn radiation methodology and tracer techniques. A two-part course is offered for the first-year trainees. One part of the course is given in didactic lecture form, and consists of a detailed survey of the body of knowledge in radiation biology as related to cancer research. The other part of the course is a survey of the techniques used in actual laboratory investigation, and is taught mainly by demonstrations. The first year trainee will be expected to spend a good part of his time in the laboratory learning the techniques which most suit his field of interest. He will work with a preceptor or a group of preceptors, and his research would be of a beginning kind. Second and third year trainees will be given the opportunity to do more advanced and independent research, employing radiation methods in their chosen fields of interest.

Applications for the training program are being accepted *until April 15, 1961* to begin July 1, 1961. All inquiries may be directed to Donald J. Pizzarello, Ph.D., Executive Director, Radiation Biology and Cancer Related Research Training Program, Bowman Gray School of Medicine, Winston-Salem, North Carolina.

## Training Program for Steroid Biochemistry

The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts with The Department of Chemistry, Clark University, Worcester, Massachusetts and The Department of Biochemistry, College of Medicine, University of Utah, Salt Lake City, Utah, announce the 1961–1962 session of the Training Program for Steroid Biochemistry.

Under the sponsorship of the National Cancer Institute, N.I.H., U.S.P.H.S., specialized programs have been established in Worcester and Salt Lake City to train investigators in the theoretical and methodological aspects of the biochemistry of steroids and related compounds. Each program includes lectures, planned laboratory work, and research.

Postdoctoral candidates (M.D. or Ph.D.) selected for admission both at Worcester and Utah will receive stipends of $5500 for a one-year period. Predoctoral candidates (B.S., M.S., or equivalent) selected for admission (only at Worcester) will receive stipends of $1800 for a six-month period.

Classes start October 1, 1961. Final date for completion of application: *June 1, 1961.*

Send inquiries and requests for applications to:

Dr. Kristen Eik-Nes,
Department of Biochemistry,
College of Medicine,
University of Utah,
Salt Lake City, Utah.

Dr. William R. Nes,
Department of Chemistry,
Clark University,
950 Main Street,
Worcester, Massachusetts.

## International Neurovegetative Symposium

The Eleventh Symposium of the International Society of Neurovegetative Research will be held in Turin, Italy, *June 11–14, 1961.* The two chief themes of the Symposium will be: "Central Nervous System Mechanisms and the Skin" and "Regulating Mechanisms in Obesity and Cachexia." All correspondence regarding general information should be addressed to Professor Dr. A. Sturm, Medizinische u. Nervenklinik, der Stadtischen Kranken-Anstalten, Wuppertal-Barmen, Germany.

## First International Colloquium on Endocrinology

The First International Colloquium on Endicrinology, organized by the Association Médicale France-Espagne and the Medical School of Barcelona, will be held *May 20–22, 1961* (Whitsuntide) in the Park-Hotel San Jorge, on the Costa Brava, some 70 kilometers north of Barcelona. The conference will consist of lectures on clinical, experimental and biochemical endocrinology delivered by eminent scientists from various European countries. A limited number of rooms have been reserved in the hotel in which the meeting will be held, for those who first indicate that they will attend. Full board and lodging, including taxes, cost only 300 pesetas per person per day—the equivalent of $5.80. All inquiries should be addressed to Dr. Joseph M. Cañadell, Avenida Generalisimo 497, Barcelona 15, Spain.

# Book Register

*Asexual Propagation and Regeneration.* By M. A. VORONTSOVA and L. D. LIOSNER. Translated from the Russian by P. M. Allen, and edited by F. Billett. Based on a course of lectures by Prof. Vorontsova to the students of the biological faculty of Moscow University, tracing the evolutionary connection between regeneration and asexual reproduction. Part I describes the processes of asexual reproduction. Part II deals with the processes of physiologic regeneration. Part III deals with the processes of reparative regeneration—in protozoa, porifera, coelenterata, platyhelminthes, nematoda, annelida, worm-like organisms, mollusca, arthropoda, echinodermata, lower chordata, and vertebrata. The last chapter concerns the general laws of the process of reparative regeneration. There is an up-to-date bibliography; 489 pages; 210 figures; 1960. Pergamon Press Inc., 122 E. 55th St., New York 22, N. Y. Price $12.00.

*Bedside Medicine.* By I. SNAPPER, M.D., Director of Medicine and Medical Education, Beth-El Hospital, and Consultant in Medicine, Veterans Administration Hospital, Brooklyn, N. Y. The author believes that the cycles in medical thinking will lead the medical profession back to Hippocratic methods reinforced by the application of modern laboratory techniques. Bedside medicine is discussed in chapters on diseases of the heart and blood vessels, esophagus, gastro-intestinal tract, liver, pancreas, respiratory tract, blood, kidney, endocrine organs, collagen system, bones and joints; there are also chapters on metabolic diseases, syndromes due to granuloma formation, fever of unknown origin, selected neurologic syndromes, and contagious and parasitic diseases; 592 pages; 1960. Grune & Stratton, 381 4th Ave., New York 16, N. Y. Price $14.50.

*Hormones in Human Plasma: Nature and Transport.* Edited by HARRY N. ANTONIADES, Ph.D., Protein Foundation Laboratories, Jamaica Plain, and Department of Gynecology, Harvard Medical School, Boston, Mass., with a foreword by GEORGE W. THORN, M.D. As stated in the foreword and the preface: "This monograph . . . is certain to fill a need . . . for an authoritative statement by experts on the problems peculiar to human function. (It) may be expected to stimulate research and integration at all levels, including, on the one hand the molecule, its configuration and physical chemistry, and on the other the patient exhibiting a serious physiological disability." "Questions as to the nature of hormones and their transport mechanism in plasma, both in normal and abnormal states, are being eagerly explored. The attempt to answer these questions has brought scientists from various fields together in a joint effort . . . working under most delicate conditions in a most complex system." Thirty-three contributors discuss the subjects under the following headings: Part I. Methods of blood collection and plasma fractionation. Part II. Hormones of pancreatic origin; insulin-like activity and anti-insulin factors in human plasma; glucagon in plasma. Part III. Hormones of pituitary origin; detection of growth hormone in plasma, adrenocorticotropic hormone, pituitary gonadotropins, thyrotropic hormone(s); vasopressin and oxytocin. Part IV. Steroid hormones; androgens in human plasma; corticosteroids in blood; estrogens in plasma, progesterone; and the binding of steroid hormones and their metabolites by plasma proteins; 667 pages; 1960. Little, Brown & Company, Boston 6, Mass. Price $25.00.

*Lipide Metabolism.* Edited by KONRAD BLOCH, Department of Chemistry, Harvard University, Cambridge, Mass. Several authorities from all over the world contribute articles dealing with the transformations of lipides by living systems and by isolated enzymes. Up-to-date information is provided for research workers in the field of metabolism and degenerative diseases. The eight chapters are entitled: Enzymatic Mechanisms of Fatty Acid Oxidation and Synthesis; The Metabolism of the Unsaturated Fatty Acids; The Metabolism and Function of Phosphatides; Metabolism of Glycerides; Lipolytic Enzymes; Hormonal Regulation of Fatty Acid Metabolism; Formation and Metabolism of Bile Acids; and Chemistry and Metabolism of Bacterial Lipides; 411 pages; illustrated; 1960. John Wiley & Sons, Inc., New York 16, N. Y. Price $10.50.

*Lipids and the Steroid Hormones in Clinical Medicine.* Edited by F. WILLIAM SUNDERMAN, M.D., Clinical Professor of Medicine, Jefferson Medical College, Philadelphia, Pa., and F. WILLIAM SUNDERMAN, JR., M.D., Instructor in Clinical Pathology, U. S. Naval Medical School, Bethesda, Md. Proceedings of an Applied Seminar of the Association of Clinical Scientists, Washington, D. C., prepared in an endeavor to assist scientists in clinical and research laboratories in selecting reliable methods of analysis and in interpreting the analytical results. Among the recent developments covered are: newer knowledge in the methodology of the analysis of phospholipids, cholesterol, unesterified fatty acids and lipoproteins; the clinical significance of unesterified fatty acids, hyperlipidemia, and electrophoresis of serum lipoproteins and lipids in cardiovascular disease; recent advances in the methodology of the steroid hormones, with emphasis on paper and column chromatography and radioisotope dilution procedures; and clinical application of measurements of 17-ketosteroids, corticosteroids, estrogens and progesterone metabolites; 207 pages; illustrated; 1960. J. B. Lippincott Company, Philadelphia. Pa. Price $10.75.

*Medizinische Grundlagenforschung. Band III.* Edited by PROF. DR. K. FR. BAUER, Erlangen. Several authorities from different parts of the world contribute articles to this large volume, in which the aim is to present a representative cross-section through the wide field of theoretical medicine as a supporting framework of clinical medicine, and to discuss problems and methods of related disciplines. (In German.) The subjects of the articles include methods of modern psychiatric research, classification of vascular abnormalities and vascular tumors of the brain, the tonus of the skeletal musculature, clinical aspects of muscle tonus, the *in vivo* measurement of radioactive substances in man, problems of protection against radiation, the protein molecule, the newest advances in the study of plasma proteins, physiologic and clinical problems of fructose metabolism, fat metabolism and atherosclerosis, capillary exchange in the higher vertebrates, the biologic significance of blood coagulation in man, the mechanism of prothrombin activation, the structure and function of synovial tissue, and our present-day knowledge of evolution; 754 pages; 205 figures; 47 tables; 1960. Georg Thieme Verlag, Stuttgart. In the U.S.A. and Canada, Intercontinental Medical Book Corporation, New York 16, N. Y. Price, DM 178; $42.40.

*Moderne Entwicklungen auf dem Gestagengebiet. Hormone in der Veterinärmedizin. Sechtes Symposion der deutschen Gesellschaft für Endokrinologie.* Edited by PROF. DR. H. NOWAKOWSKI, II. Med. Univ.-Klinik und Poliklinik, Hamburg-Eppendorf, Germany. (In German.) The Symposium was held in Kiel, April 28–30, 1959. Many authoritative participants present papers on various aspects of gestation,

and the sex hormones, including a special section on hormones in veterinary medicine. Part I concerns the newer knowledge of gestagens in the human organism, the use of radioactive isotopes in research, the ovariectomized woman, the influence of nortestosterone on the pituitary-hypothalamic system, the cytostatic effect of various progestins, basal body temperature, progesterone therapy, and gonadotropins. There is another section on the androgens in the ovary. Part IV pertains to a wide range of endocrinologic problems, in man as well as in animals, including data on insulin, the corticosteroids, oxytocin, gonadotropins, androgens and estrogens; and some clinical syndromes; paper; 441 pages; 276 illustrations; 1960. Springer-Verlag, Berlin-Wilmersdorf, Heidelberger Platz 3 (West-Berlin), Germany. Price, DM 96.

*Sea Within. The Story of Our Body Fluid.* By WILLIAM D. SNIVELY, JR. M.D.,, Lecturer in Pediatrics, University of Louisville School of Medicine. The author states that the book is intended primarily for the great number of laymen who are intrigued by the scientific aspects of human life. It may also be of interest to physicians, nurses, technicians, dietitians, and students as background reading for technical treatises on body fluid. It provides an unusual synthesis of science and the humanities, written in readily understandable terms, from the first chapter entitled "In the Beginning" to the last chapter entitled "The Vast Grey Seas Ahead"; 150 pages; 55 figures; 1960. J. B. Lippincott Company, Philadelphia, Pa.

*Transactions of the Josiah Macy, Jr. Foundation Conferences.* Publications Office, 16 West 46th Street, New York 36, N. Y.

*Genetics.* First Conference, October 19–22, 1959, Princeton, N. J. Edited by H. ELDON SUTTON, Ph.D., Department of Medical Genetics, University of Michigan Medical School, Ann Arbor, Michigan. The material is considered under three main headings: 1) Current concepts of the fine structure of chormosomes and the nature of the coding mechanism. 2) The genetic control of protein structure. 3) Factors modulating the biochemical expression of genetic systems; 229 pages; 38 figures; 6 tables; 1960. Price $6.00.

*The Central Nervous System and Behavior.* Third Conference, February 21–24, 1960, Princeton, N. J., with the cooperation of the National Science Foundation, Washington, D. C. Edited by MARY A. B. BRAZIER, D.Sc., Neurophysiological Laboratory, Massachusetts General Hospital, Boston, Mass. The subjects include: The evolution of man's brain, biochemical maturation of the central nervous system, neuronal models and the orienting reflex, concepts of drive, affectional behavior in the infant monkey, verbal regulation of behavior, and signaling systems in the development of cognitive functions. There is a list of Russian articles translated in preparation for the Conference; 475 pages; 201 figures; 1960. Price $7.50.